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(19)(CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) DNA Molecule Encoding Freezing Tolerance Proteins in Gramineae

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ABSTRACT OF THE DISCLOSURE

The present invention relates to three novel genes which have been isolated from cold-tolerant wheat species and which are induced by low temperature. The first gene, Wcs19, is preferentially expressed in green leaf tissues of tolerant gramineae species and requires both light and low temperature for maximal induction. The second gene, Wcs120, is induced only by low temperature. Different from the protein encoded by Wcs19, the protein encoded by Wcs120 contains two repeated domains that are highly conserved among RAB (rice abscisic acid-induced) and dehydrin families and appears to be light-independent. The Wcs120 protein does not however contain a serine-rich sequence present in RAB and dehydrin families. Finally, the present invention also relates to a third gene, Wcor410, also induced by low temperature as well as water stress and, to a lesser extent, by ABA. Its expression is light-independent. The protein encoded by this gene contains a serine-rich stretch as found in several drought induced proteins.

TITLE OF THE INVENTION

DNA molecules encoding freezing tolerance proteins in gramineae

BACKGROUND OF THE INVENTION

Wheat is a temperate cereal that possesses the capacity to develop a high degree of freezing tolerance (FT) following a period of low temperature growth. In general, winter cultivars, compared to spring ones, possess better protective mechanisms allowing them to optimize growth at low temperature while the induction of FT takes place. This difference is genetically programmed and provides winter cultivars with a competitive advantage to grow at low temperatures. This results in lengthening the growing season since the plant is able to capitalize on favourable conditions that may occur late in the season. Understanding the complexity of this genetic system and its regulation by environmental factors leading to the increase of FT is still a challenge. Development of FT in plants is a metabolically active process induced by low temperature and is associated with altered gene expression [13,42]. Several proteins and their corresponding mRNAs accumulate during cold acclimation. In certain cases, their accumulation was associated with the capacity of plant and tissues to develop FT [15, 31]. Some of these

genes are specifically upregulated by low temperature [14, 25, 30, 46] while others are also induced by other factors such as abscisic acid (ABA) and water stress [20, 22, 24]. Sequence analysis of some of these genes has not revealed any information that improves our understanding of their function. Unlike heat shock, water or salinity stresses, FT is not associated with a universal response. The proteins that accumulate during cold acclimation were first believed to be species-specific but recent results indicate that they are family-specific [15, 46]. We have identified a wheat protein family which is upregulated specifically by low temperature and found it to be expressed only in freezing tolerant gramineae species [15]. The kinetics of accumulation and the abundance of these proteins during cold acclimation suggest a close relationship between the development of FT and the amount of these proteins [15]. However, their exact function in FT remains to be established. Since FT is a multigenic trait, the isolation of all the genes involved is required to understand the overall genetical and physiological bases regulating the process of cold acclimation and the induction of FT.

STATEMENT OF THE INVENTION

The present invention relates to a first novel gene regulated specifically by low temperature and associated with the leaf development. This gene, Wcs19, is preferentially expressed in green leaf tissues of tolerant gramineae species and requires both light and low temperature for maximal induction. A second gene has also been sequenced. This gene, Wcs120, encodes a protein which is also induced by low temperature. This gene, also under the scope of the present invention, is very weakly induced by water stress and ABA. Different from the protein encoded by Wcs19, the protein encoded by Wcs120 contains two repeated domains that are highly conserved among RAB (rice abscisic acid-induced) and dehydrin families and appears to be light-independent. The Wcs120 protein does not however contain a serine-rich sequence present in RAB and dehydrin families. Finally, the present invention also relates to a third gene, Wcor410, also induced by low temperature as well as water stress and, to a lesser extent, by ABA. Its expression is light-independent. The protein encoded by this gene contains a serine-rich stretch as found in several drought induced proteins.

DESCRIPTION OF THE INVENTION

Plant material and growth conditions

In this study we used three wheat genotypes: spring wheat (Triticum aestivum L. cv Glenlea, LT_{50} -8°C) and winter wheat (T. aestivum L. cv Fredrick, LT_{50} -16°C, cv Norstar, LT_{50} -19°C), winter rye (Secale cereale L. cv Musketeer, LT_{50} -21°C), barley (Hordeum vulgare L. cv Winchester, LT_{50} -7°C), oat (Avena sativa L. cv Laurent, LT_{50} -3°C), rice (Oriza sativa, LT_{50} 4°C), alfalfa (Medicago falcata cv Anik, LT_{50} -12°C, canola (Brassica napus cv Jet neuf, LT_{50} -16°C). FT was determined as reported previously [31] and expressed as the temperature required to kill 50% of the seedlings (LT_{50}).

Seeds were germinated in moist sterilized vermiculite for 5 days in the dark and 2 days under artificial light at 25°C/20°C (day/night) with a 15 h light period at an irradiance of 250 $\mu mol.m^{-2}.s^{-1}$. Control plants were maintained under the same conditions while cold acclimation was performed by subjecting the seedlings to acclimation conditions (6°C/2°C day/night, 10 h photoperiod). In the case of rice exposed to low temperature, the day/night cycle was of 10°/5°C. For ABA treatment, 7-day old seedlings were watered daily for 4 days with nutrient solution containing 10^{-5} M ABA.

As expected, ABA-treated plants showed a reduced growth rate compared with the control, indicating that ABA elicited the proper physiological response. Water stress was induced by withholding water until the plants became visibly wilted (4 days). Heat shock was performed by incubating seedlings at 40°C for 3 h. This treament was sufficient to induce typical heat shock proteins as described in our previous work [7]. Salt-stressed plants were grown for 18 h in solutions containing 500 mM NaCl. Anaerobic treatment was accomplished by submerging seedlings under water in an airtight container for 24 h. Wounding was performed by slicing leaves to 1 cm pieces and floating on water for 14 h. Etiolated seedlings were grown in complete darkness. Deacclimation was performed by returning cold-acclimated plants (36 days at 4°C) to normal growth conditions for 1 and 5 days.

Preparation of calli

The calli used in this study were derived from winter wheat (T. aestivum L. cv Fredrick). Plants were grown under the same environmental conditions mentioned above, using a mixture of soil:peat:vermiculite (1:1:1;v/v/v) supplemented with a soluble fertilizer (N:P:K 20:20:20). Inflorescences were tagged at the

onset of anthesis and the spikes were harvested 12 days postanthesis. The kernels were surface-sterilized as described earlier [4]. The embryos were aseptically isolated and cultured on Murashige and Skoog [27] (MS) medium supplemented with 30 $g.l^{-1}$ sucrose, 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.8% Difco Bacto Agar (pH 5.7). The cultured plates were incubated at 24/20°C with a 15 h photoperiod under low irradiance (100 $\mu mol.m^{-2}.s^{-1}$). The callus cultures were maintained by subculturing every 2 weeks on the same medium. Low temperature exposure of calli was done at 6/2°C (day/night).

Construction and screening of the cDNA library

Poly(A)$^+$ RNA was isolated from cold-acclimated winter wheat Norstar [8]. A cDNA library was constructed in lambda ZAPII (Stratagene) using EcoRI-Not I linkers from Pharmacia, and transformed into Escherichia coli strain XL-1 blue using techniques well known in the art. The subtractor kit from Invitrogen was used in accordance with manufacturer's directives to generate a cDNA subtracted probe, prepared from poly(A)$^+$ RNA isolated from cold-acclimated and non-acclimated winter wheat plants. The plaques showing an increased hybridization signal with the subtracted probe or by

differential screening (14) (clones pWcs19, pWcor410 and pWcs120) were selected and purified, and subcloned via the automatic excision process described by Stratagene. The screening of the library and all the recombinant DNA techniques were performed using techniques well known in the art [36].

Northern and Southern blot analyses

Poly(A)$^+$ RNA (4 μg) or total RNA (10 μg) samples were mixed with ethidium bromide before electrophoresis on formaldehyde agarose gels [35]. This allowed visual evaluation of RNA quality and loads on gels. A clone previously isolated, p2.1, that did not display differential hybridization during cold acclimation was also used to verify the equal loading of RNA. After electrophoresis, RNA or DNA was transferred to nitrocellulose membranes (BAS-85, Schleicher & Schuell) in 20 X SSC. The filters were air-dried and then baked for 1 h at 80°C prior to hybridization with the ^{32}P-labelled pWcs19, pWcor410 or pWcs120 inserts [33]. Filters were washed at 65°C with several buffer changes of decreasing SSC concentration (5 to 0.1 X) and then autoradiographed on Kodak XRP films with intensifying screens (DuPont, Cronex Lightning plus) at -80°C.

Genomic DNA was prepared from shoots of 8-day old seedlings as previously described [34], and DNA samples (10 μg) were digested with appropriate restriction endonucleases prior to electrophoresis.

DNA sequence analysis

Plasmid DNA was prepared, and deletion subclones were generated using exonucleases III and VII, as described [48]. Plasmids were sequenced by the dideoxynucleotide chain-termination method [37] with the aid of T7 and Gene-ATAQ kits from Pharmacia. Sequence comparison was carried out with the Genetic Computer Group's Sequence Analysis Sofware package, version 6.0, with a Vax computer (Université de Montréal). The database was searched with the TFASTA program. The hydropathy profile was calculated according to Kyte and Doolittle [18], using a 6 aa window. Secondary structure predictions were made by the method of Garnier et al. [11].

In vitro transcription/translation

pWcs19: pWcs19 was linearized by digestion with Hind III and the coding strand was transcribed in vitro with T3 RNA polymerase. The reaction volume of 20 μl contained 5 mM MgC12, 50 mM Tris-HCI pH 7.5, 10 mM DTT,

2 mM spermidine, 10 U of RNase inhibitor, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 0.5 mM UTP, 500 ng of linearized plasmid and 40 U of T3 RNA polymerase (Pharmacia). Nucleic acids were extracted with phenol: chloroform: isoamyl alcohol (25:24:1) and precipitated with ethanol. The pellet was resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and translated in vitro using a wheat germ extract from Promega in presence of ^{35}S-methionine, according to manufacturer's directives. Translation products were fractionated on SDS-PAGE, dried and exposed to Kodak XOMAT-AR film.

pWcs120: Plasmid pWcs120 was mutated using polymerase chain reaction to introduce a NdeI restriction site at the ATG start codon and a BamHI site just after the stop codon. For this purpose, two oligonucleotides were synthesized using the Gene Assembler from Pharmacia. The first oligonucleotide, 5'-AGTGAGGATCCCAGCGCCATATGGAG AAC-3', was homologous to the coding strand of Wcs120 with the exception of four nucleotides to introduce BamHI and NdeI sites. The second oligonucleotide, 5'-GTTGTCCGGTGGATCCTTAAAC-3', was complementary to the coding strand with the exception of three nucleotides to produce a BamHI site. Amplification using the TaqI DNA polymerase (Perking-Elmer Cetus corporation) and

subcloning into pUC9 (IBI) and pET (Novagen) vectors was performed. The inserted, amplified fragment was then digested with NdeI and BamHI and ligated into NdeI-BamHI-digested plasmid pET11a. This placed the entire coding frame, including the start methionine codon, directly downstream of the T_7 promoter to allow a high level of expression in E. coli. Expression was performed in BL21 (DE3) (Novagen). At a bacterial density measured by the value of absorbance at 600 nm (A_{600}) of 0.6, 1 mM IPTG was added to the bacterial suspension, and 3 h later the bacteria were collected by centrifugation and resuspended in 0.1 volume of electrophoresis buffer for analysis.

To purify the expressed protein, bacterial cells were suspended in 5 to 10% of the culture volume of 50mM Tris (pH 8.0), 10 mM EDTA. After one freeze-thaw cycle, the cells were disrupted by sonication and the lysate was centrifuged at 15,000g for 20 min to eliminate insoluble proteins. The boiling-stable proteins were precipitated from the supernatant with ice-cold acetone and collected by centrifugation. The proteins were solubilized in electrophoresis buffer and separated on a 10% preparative polyacrylamide gel. The expressed protein was excised and electroeluted for 3 h. The eluted protein was then precipitated with acetone and

analyzed by two-dimensional gel electrophoresis as previously described.

To compare the E. coli-expressed protein with that synthesized in vitro, Poly(A)$^+$ RNA from cold-acclimated and non-acclimated wheat were translated in a wheat germ system and analyzed by two-dimensional gel electrophoresis as previously described.

The cDNA clones Wcs19, Wcor410 and Wcs120 were identified from cold-acclimated wheat shoots of the cultivar Norstar. Based on reprobing the library with the purified inserts, the representation was estimated to be 0.02 % and 0.1% for Wcs19 and for Wcs120, respectively. The isolated clones hybridize preferentially to mRNAs of 1.0, 1.3 and 1.65 kb, for Wcs19, Wcor410 and Wcs120, respectively, that accumulate upon exposure to low temperature.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1. Kinetic analysis of Wcs19 mRNA expression during cold acclimation. Poly (A)$^+$ RNA (4 μg per lane) was separated and transferred to nitrocellulose membranes as described in M & M and hybridized with ^{32}P-labelled cDNA insert from pWcs19. NA_7 and NA_{12}, control plants (non-acclimated) grown for 7 and 12 days at 24°C; A_1, A_6

and A_{36}, plants cold-acclimated for 1, 6 and 36 days; D_1 and D_5, cold-acclimated plants (36 days) were deacclimated for 1 and 5 days at 24°C. Formaldehyde gels were visualized with ethidium bromide and the clone p2.1 was used to control the equal loading and quality of RNA (not shown). A. Winter wheat Fredrick, B. Winter wheat Norstar, C. Spring wheat Glenlea.

Fig. 2. Northern blot analysis of mRNA hybridized with Wcs120 during cold acclimation and deacclimation of wheat. Poly(A)$^+$ RNAs (4 μg) isolated from nonacclimated, cold-acclimated, and deacclimated wheat plants were separated by agarose gel electrophoresis in the presence of formaldehyde and then transferred to nitrocellulose membranes. The blots were probed with ^{32}P-labelled cDNA insert from plasmid pWcs120. The final wash was at 55°C in 0.1 X SSC containing 0.1% SDS. Bands were visualized by autoradiography. A control probe (p2.1) was used to verify the equal RNA loading on the gel. NA_7 and NA_{12}, control plants (nonacclimated grown for 7 and 12 d; A_1, A_6, and A_{36}, plants cold acclimated for 1, 6 and 36 d; D_1 and D_5, plants deacclimated for 1 and 5 d. The plants used in the deacclimation experiment had been cold acclimated for 36

d. A, Glenlea; B, Fredrick,; C, Norstar. Exposure was for 2 h.

Fig. 3. Northern blot analysis of total RNA (10 μg per lane) from wheat plants (cv Fredrick) exposed to different treatments. A. The filter was hybridized with Wcs19. 1, plants cold-acclimated for one day; 2, plants water-stressed for 4 days; 3, plants treated with 10^{-5} M ABA for 4 days; 4, non-acclimated plants grown at 24°C; 5, plants heatshocked for 3 h at 40°C. B. The filter was hybridized with Wab1, an ABA-responsive clone isolated from wheat in our laboratory. Lanes 1 to 4 as in A. C. The filter was hybridized with HSP70B cDNA (StressGen, Victoria, B.C., Canada). 1, plants grown at 24°C; 2, plants heat-shocked for 3 h at 40°C.

Fig. 4. Northern blot analysis of mRNA hybridized with Wcs120 after different treatments. Poly(A)$^+$ RNAs (4 μg) isolated from plants grown as described were separated and probed as described in Figure 2. ABA, plants treated with ABA for 4 d; DR, water-stressed plants visibly wilted; HS, plants treated at 40°C for 3 h. Other symbols as in Figure 2. Exposure was for 20 h.

Fig. 5. Southern blot analysis of wheat genomic DNA. Wheat DNA from three genotypes was digested with 6 different restriction enzymes, separated by agarose gel electrophoresis, transferred to nitrocellulose and then probed with Wcs19. X, Xba 1; S, Sac 1; P, Pst 1; K, Kpn 1; H, Hind III; B, Bam H1. A, Glenlea; B, Norstar; C, Fredrick.

Fig. 6. Southern blot analysis of wheat genomic DNA. Wheat DNA (10 μg) from three genotypes was digested with 12 different restriction enzymes, separated on agarose gel electrophoresis, transferred to nitrocellulose, and then probed with Wcs120. Lane 1, ApaI; lane 2, KpnI; lane 3, SacI; lane 4, HindIII; lane 5, PstI; lane 6 PvuII; lane 7, BamHI; lane 8, EcoRI; lane 9, XbaI; lane 10, SalI; lane 11, XhoI; lane 12, SmaI. A, Glenlea; B, Fredrick; C, Norstar.

Fig. 7. DNA sequence and deduced amino acid sequence of Wcs19. The coding strand has been determined using T3 and T7 RNA transcription of Wcs19 in the Bluescript vector (Stratagene) and subsequent hybridization to RNA from acclimated plants. The longest ORF is shown here (570 nucleotides). The consensus polyadenylation signal is shown as a double underline; proline residues are

boxed; acidic residues (D and E) are circled. On a third line, secondary structure predictions were made by the method of Garnier et al. [11]. Symbols are: α, alpha helix; β, beta sheet; ⌋, turns; ϖ, random coil. GenBank Accession No.: L13437.

Fig. 8. Nucleotide and deduced amino acid sequence of Wcor410. The initiating and terminating codons are underlined.

Fig. 9. Nucleotide and deduced amino acid sequence of the Wcs120. The DNA sequence was obtained on both strands by the chain termination method. Domain A (repeated 6 times) is underlined, and domain B (repeated 11 times) is boxed. GenBank Accession No.: M93342.

Fig. 10. In vitro transcription/translation of pWcs19. The labelled translation products were separated on SDS-PAGE and visualized by autoradiography. T, translation products of the linearized vector alone; Wcs19, translation products of the linearized pWcs19, TB and Wcs19B, the translation products were boiled for 10 min.

Fig. 11. SDS-PAGE analysis of proteins present in E. coli transformed with plasmid pEWcs120. Lane 1, non-transformed; lane 2, transformed and induced with 1 mM IPTG; lane 3, nontransformed boiled extract; lane 4, transformed and induced boiled extract; lane 5, induced purified protein. The proteins were analyzed on a 10% polyacrylamide gel and visualized by staining with Coomassie blue R-250. The arrow indicates the presence of the 50-kD protein in pEWcs120-transformed cells induced with IPTG. The molecular mass markers are shown on the right side (kD).

Fig. 12. Two-dimensional gel electrophoretic analysis. A, In vitro translation products of mRNAs isolated from control (nonacclimated) winter wheat Fredrick. B, In vitro translation products of mRNAs isolated from cold-acclimated winter wheat Fredrick. Circle indicates the 50 kD-protein induced during cold acclimation. C, Purified protein expressed in E. coli transformed with pEWcs120. The protein had identical molecular weight and isoelectric point as that synthesized in vitro and in vivo.

Fig. 13. Tissue and species specificity of Wcs19 expression. Total RNA (10 μg per lane) was isolated

from the different tissues. Plants and calli were cold-acclimated for 6 days. Panel A. lanes A, B and C, root, crown and leaf tissues of cold-acclimated wheat tcv Fredrick); lane D, non-acclimated wheat leaves; lanes E and F, cold-acclimated and nonacclimated wheat calli. G to M, cold-acclimated species. Lanes: G, Brassica; H, alfalfa; I, rice; J, rye; K, barley; L, oat; M, wheat. Lanes N to T correspond to the non-acclimated tissues of G to M respectively. Panel B. Ethidium bromide-stained gel.

Fig. 14. Light requirement for the expression of Wcs19. Total RNA (10 μg per lane) was isolated from etiolated wheat seedlings (cv Fredrick) cold-acclimated in the dark (lane 1) or in the presence of light (lane 2) for 4 days. A, ethidium bromide-stained gel; B, the filter was hybridized with Wcor410, a cold-regulated clone isolated in our laboratory; C, the filter was hybridized with Wcs19.

Fig. 15. Immunoblot characterization of anti-Wcs120 antibody. A. Purified Wcs120 protein used for immunization. P, preimmune serum, I, affinity purified immune serum. B. Lane 1, soluble proteins from non-acclimated wheat (cv. Fredrick) seedlings; lane 2, 36

day cold-acclimated wheat seedlings. C. Proteins were isolated from winter wheat cv. Fredrick cold-acclimated for 36 days, and separated on a 2D gel. After transfer to nitrocellulose, the filter was incubated with the anti-Wcs120 antibody and processed. Several proteins in the basic portion of the gel.

Fig. 16. Northern Blot analysis of winter wheat cv Fredrick mRNA after cold acclimation and deacclimation. Poly $(A)^+$ RNAs (4 μg) isolated from non-acclimated, cold-acclimated, and deacclimated wheat plants were separated by agarose gel electrophoresis in the presence of formaldehyde and then transferred to a nitrocellulose membrane. The blots were probed with ^{32}P-labelled cDNA insert from clone pWcs120 and bands were visualized by autoradiography. One major band of 1.7 kb and four minor bands varying in size from 0.8 to 5 kb were detected. NA_7 and NA_{12} non-acclimated 7 and 12 days plants; A_1, A_6 and A_{36}, cold-acclimated for 1, 6 and 36 days; D_1 and D_5, deacclimated for 1 and 5 days. The plants used in the deacclimation experiment had been cold-acclimated for 36 days.

Fig. 17. Accumulation kinetics of freezing tolerance markers (FTMs) in Triticum Aestivum L. cv. Fredrick and

cv. Glenlea. A. Coomassie Blue-stained gel of cv. Fredrick. B. Parallel gel transferred to nitrocellulose and probed with the purified anti-Wcs120 antibody. C. Coomassie blue-stained gel of cv. Glenlea; 50% more proteins were loaded compared with A. D. Parallel gel transferred to nitrocellulose and probed with the purified anti-Wcs120 antibody. NA_{12} non-acclimated 12 day old plants; A_1, A_6 and A_{15} and A_{36}, cold-acclimated for 1, 6, 15 and 36 days; D_1 and D_6, deacclimated for 1 and 6 days. The plants used in the deacclimation experiment had been cold-acclimated for 36 days. High molecular weight markers (Bio-Rad) are shown on the left side.

Fig. 18. Immunoblot analysis of FTMs isolated from different tissues and from plants grown in different conditions. A. Proteins isolated from different plant tissues (cold acclimated cv. Fredrick) and probed with the purified anti-Wcs120 antibody. Lane 1, root; lane 2, basal region of the crown; lane 3, meristematic crown; lane 4. leaf. B. Effect of different growth conditions on FTMs isolate from shoot tissue. Lane 1, Triticum aestivum L. cv. Fredrick cold-acclimated for 36 days; lane 2, salt-treated cv. Fredrick; lane 3, water-stressed cv. Fredrick; lane 4, abscisic acid-treated cv.

Fredrick; lane 5, heat-shocked cv. Fredrick; lane 6, Hordeum vulgare L. cv. Winchester cold-acclimated for 36 days; lane 7, water-stressed cv. Winchester.

Fig. 19. Immunoblot analysis of soluble proteins isolated from different cold-acclimated species. Lane 1, Triticum aestivum L. cv. Fredrick; lane 2, Agropyron repens L.; lane 3, Secale cereale L. cv. Musketeer; lane 4, Hordeum vulgare L. cv. Winchester; lane 5, Avena sativa L. cv. Laurent; lane 6, Phleum pratense L.; lane 7, Zea mays; lane 8, Oryza sativa; lane 9, Brassica napus L.; lane 10, Mentha canadensis; lane 11, Petunia hybrida; lane 12, Medicago falcata cv. Anik.

RESULTS

The kinetic studies using northern blot analysis (Figs. 1 and 2) show that the accumulation of Wcs19 and Wcs120 is very rapid, and remains at a constant level throughout the acclimation period in both freezing tolerant cultivars, Fredrick and Norstar. On the other hand, in the less tolerant cultivar Glenlea, the expression of these mRNAs declines despite maintaining the plants at 4°C. When the plants were deacclimated at 24°C, the steady-state level of Wcs19 and Wcs120 transcripts declined rapidly. Figs. 3 and 4 show that

the Wcs19 and Wcs120 mRNAs were not induced by water stress, exogenous ABA application or heat shock. Positive controls were used to demonstrate that the treatments elicited the typical molecular responses in addition to the physiological ones described above. Other treatments such as wounding, anaerobic and salt stresses did not show any effect on the expression of Wcs genes (not shown). These results indicate that the accumulation of Wcs19 and of Wcs120 is specifically induced by low temperature. The expression of Wcor410 (results not shown) is regulated by low temperature in freezing tolerant monocots only and correlates with the capacity of plants and varieties to develop freezing tolerance. This gene was also induced to a similar level by water stress and to a lesser extent by ABA.

Southern analysis, shown in Figs. 5 and 6, using probes Wcs19 and Wcs120, did not reveal any differences in the restriction pattern between the three genotypes. This result suggests that the reduced expression of Wcs19 and of Wcs120 in the less tolerant cultivar is not due to a detectable difference in the genomic organization of the gene or in the relative gene copy number since the bands intensities are comparable.

Sequence analysis

The complete DNA and amino acid sequences of Wcs19, Wcor410 and Wcs120 are shown in Figs. 7, 8 and 9. Because of the degeneracy of codons, it is understood that any DNA sequences encoding the same amino acid sequences are under the scope of the invention. It is also understood that conserved amino acid substitutions in similar sequences as determined by techniques well known in the art are under the scope of this invention. Such conservative substitutions are frequently encountered, when comparing sequences of molecules which have diverged from a common ancestor. In different varieties of a same species, for instance in T. aestivum L. cv Winoka (a variety of wheat), conservative substitutions are encountered in a proteic sequence closely related to pWcs120 protein [50]. Long open reading (ORF) frames were found in both DNA orientations. Northern blots were thus probed with labelled RNAs produced by in vitro transcription of the inserts using the T3 or T7 promoters and corresponding RNA polymerases. The predicted polypeptides encoded by Wcs19, Wcor410 and Wcs120 are 190, 262 and 390 amino acids in length and have a calculated molecular mass of 19, 28 and 39 kDa and a pI of 8.8, 5.1 and 7.7, respectively.

Wcs19: Search of the Genbank database revealed no homology with any protein. However, at the DNA level, a significant homology was found with a cold regulated partial DNA sequence (pT59) from barley [3]. The sequence analysis indicates that the protein is alanine-rich (21%) and has high content of glycine (8%), lysine (8%) and proline (7%). These four amino acids represent 44% of the polypeptide. The protein has a particular charge distribution. The acidic amino acids aspartate and glutamate (Fig. 7, circled) are localized towards the C-terminal half and thus give this region a net negative charge of -7. Furthermore, this region (from aa 100 to the end) has a high propensity to form an alpha helix as observed for some transcription factors [32]. The N-terminal half is rich in proline (boxed), lysine and arginine, and has a net positive charge of +10. The hydropathy profile (not shown) indicates that most of the protein is hydrophilic except between amino acids 42 and 59.

Wcor410: This protein is rich in glutamate (16%) and has a compositional bias for charged amino acids (43%). It also contains a succession of 9 serine residues as found in several drought induced proteins (see Fig 8).

Wcs120: The predicted protein has a compositional bias for Gly (26.7%), Thr (16.7%), and His (10.8%). These three amino acids account for 54% of the polypeptide, whereas Cys, Phe, and Trp are absent. The predicted protein contains two repeated domains. The A repeat is basic and has the consensus sequence GEKKGVMENIKEKLPGGHGDHQQ, which is repeated six times in the ORF (underlined in Fig. 9). The B repeat contains 14 amino acids (consensus sequence TGGTYGQQGHTGTT) and is repeated 11 times.

A computer search revealed that repeats A and B are found in the dehydrin and RAB protein families, with the exception that the B repeat was not found in dehydrins 8 and 9. Outside these repeats, very little homology was found between Wcs120 and these two protein families with the exception that all the predicted polypeptides start with ME and, at the carboxy tail, share a stretch of 18 highly conserved amino acids ending with QH. These similarities suggest that Wcs120 could share a function with the dehydrins and RAB families. On the other hand, the conserved sequence SGSSSSSSS, found in all RAB and dehydrin proteins, was conspicuously absent in Wcs120.

In vitro transcription/translation experiments with Wcs19 were performed as described above. Fig. 10 shows

a specific translation product of 26 kDa (lane Wcs19). The apparent MW of 26 kDa is higher than the predicted mass of 19 kDa. This discrepancy has already been observed for several other stress proteins [14, 45]. Since three other ATG start codons present in the 5' region of the cDNA are followed by in frame stop codons, we determined whether the longest ORF identified was able to encode the same protein. This ORF was subcloned using the polymerase chain reaction and the transcription/translation experiment was repeated with the subclone. The same translation product size was obtained indicating that the ATG at position 205 is the first one that can be used and that the stop codons identified before this ATG cannot be due to sequencing errors. The proper stop codon is found at position 775, and the consensus polyadenylation signal is found at position 924. The product of translation does not remain in solution after boiling as shown in lane Wcs19B and is thus different from other alanine- and glycine-rich stress proteins cloned up to date [5, 14, 16, 20, 30].

To identify the Wcs120 encoded protein, the Wcs120 ORF was expressed in E. coli. The protein was purified from E. coli (Fig. 11) and compared with the in vitro translation products of RNA isolated from nonacclimated

and cold-acclimated plant (Fig. 12). The protein synthesized by bacteria was boiling stable and co-migrated with a 50-kD protein produced by mRNAs isolated from the cold-acclimated plants. The protein produced in the bacteria also co-migrated with a protein that accumulated in vivo during cold acclimation, suggesting that little or no posttranslational modifications were occurring in the intact plants and that, in both species, the first initiation codon, at position 23, was probably used. A discrepancy between the calculated and apparent molecular masses on SDS-PAGE was observed (39 versus 50 kD). This discrepancy likely results from the avid binding of SDS, as suggested for other plant stress proteins with skewed amino acid composition.

Tissue and species specificity

Northern blot analyses of Wcs19 mRNA expression in root, crown and leaf tissues of acclimated wheat seedlings are shown in Fig. 13. The results indicate that the Wcs19 expression is specific to leaf tissue. The expression was hardly detected in root and crown. To determine if the expression is strictly associated with the leaf structure, the expression of the gene in non-differentiated tissues such as wheat calli was compared to that of leaves. The results in Fig. 13,

lane E, show that no accumulation of mRNA occurs during calli acclimation to low temperature. Furthermore, there was no detectable expression in other tissues such as the flower parts, developing or mature embryos (not shown). These results demonstrate that the Wcs19 mRNA accumulation is leaf-specific and is not directly needed for the acquisition of FT in other tissues. The accumulation of Wcs19 mRNA was found to be present in most cereal species that can cold acclimate and suggests that it may play a crucial role in the leaf acclimation to low temperature. The highest levels of accumulation was found in the most tolerant species, wheat and rye, compared to that of barley, a less tolerant species. Oat and rice did not show any accumulation of Wcs19. On the other hand, the two dicot tolerant plants examined, Brassica and alfalfa did not show any induction. This indicates that the Wcs19 is gramineae-specific and that its expression is correlated to the capacity of each genotype to develop FT.

On the other hand, when using an antibody anti-Wcs120, it appears that freezing tolerance markers are retrieved in leaves as well as in crown and roots. The more tolerant parts (leaves and meristematic crown) contain more of these markers than less tolerant parts of plants (roots and basal region of the crown). This

could mean that tissue-specificity of Wcs120 is different from Wcs19 or that the antibodies raised against Wcs120- encoded protein can recognize the protein Wcs120 and other related proteins which distribution is different from Wcs19.

Light requirement

The association of Wcs19 expression to the leaf tissue and its complete absence in the non-photosynthetic tissues drew our attention to the possibility that light may be required for expression. The results in Fig. 14 show that light is required for maximal mRNA accumulation since etiolated plants accumulated at least 4 fold less Wcs19 transcripts. The cold-regulated gene Wcor410 was not affected by the presence or absence of light (fig. 14B). This result confirms the light stimulation of Wcs19. In addition, as for cold-acclimated callus cultures, albino seedlings lacking chloroplastic structures and chlorophyll (generated from some callus cultures) were not able to accumulate any detectable amount of Wcs19 transcript in the presence of light and low temperature (not shown). These results indicate that the Wcs19 expression is dependent on organized leaf tissue and that light acts as a stimulating factor.

When using antibodies anti-Wcs120, the expression of the freezing tolerance markers was not limited to photosynthetic tissues and was not light-dependent.

Development of antibodies to select for freezing tolerance

Antibody production and purification

Antibodies against Wcs120 were generated using antigen synthesized in E. coli [14]. The purified protein was used to generate antibodies in a New Zealand rabbit, Preimmune serum was taken from the rabbit before the first immunization and immune serum was taken 10 days after the second and subsequent injections.

For antibody purification, the purified Wcs120 protein was coupled to Affi-gel 10 (Bio Rad) at 3 mg/ml of bed resin in 0.1 M HEPES buffer, pH 7.5 containing 80 mM $CaCl_2$. The coupling was performed at 4°C overnight. Free sites were saturated with 0.2 M ethanol-amine for 1 h. The coupled resin was washed with phosphate buffered saline (PBS) containing 0.1% NP-40 and the serum was incubated for 1 h with the beads. After washing with PBS, the bound antibodies were eluted with 0.3 M glycine, pH 2.0 and immediately neutralized with TRIS base. The purified antibodies were then dialyzed against PBS and lyophilized.

Protein extraction, separation and immunoblot analysis

Soluble proteins were extracted from different tissues by grinding in a precooled mortar with TRIS buffer [0.1 M TRIS-HCl, pH 9.5 containing 1 mM phenylmethylsulfonyl fluoride (PMSF)]. The extract was immediately centrifuged for 5 min at 12000 g and the supernatant was adjusted to final buffer concentration with 5 X SDS electrophoresis sample buffer [19]. Samples were separated by electrophoresis on 10% polyacrylamide-SDS gels (SDS-PAGE) or on two-dimensional (2D) gels as described [8].

Total soluble proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose (BAS-85, Schleicher and Schuell). After blocking with powdered milk (2%) in PBS containing 0.2% Tween-20 (Blotto), the blot was incubated with a 1:1000 dilution of the purified Wcs120 antibody. After washing with PBS-Tween, the proteins recognized by the primary antibody were revealed with alkaline phosphatase coupled to anti-rabbit IgG as secondary antibody. The complex was revealed by incubating in 100 mM TRIS, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.2 mg/ml nitroblue tetrazolium, and 0.2 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate.

Northern blot analysis

RNA isolation and Northern blot analysis were as previously described [14] except that washing stringency was lowered to 5 X SSC at 55°C.

Specificity of anti-Wcs120 antibody

The purified Wcs120 protein produced in the bacterial system and proteins extracted from control and cold acclimated wheat seedlings were separated by SDS-PAGE and transferred to nitrocellulose. Preimmune serum did not react with the Wcs120 protein while the purified anti-Wcs120 antibody recognized the 50 kDa protein expressed in E. coli and that synthesized in cold acclimated seedlings (Fig 15). No proteins were recognized in the control non-acclimated plant. The anti-Wcs120 antibody also recognized several other proteins from cold acclimated seedlings. Analysis by 2D gel electrophoresis (Fig. 15C) revealed that they were neutral to slightly basic (from pI 6.5 to 7.3) and correspond to proteins previously identified during cold acclimation by in vivo labelling of proteins and by in vitro translation of mRNA isolated from cold acclimated plants [8].

Northern blot analysis of Wcs120 related mRNAs

The anti-Wcs120 antibody identified at least four protein groups on 1D and 2D gels. In order to examine whether this homology was apparent at the mRNA level, we probed a Northern blot with the pWcs120 insert and washed it at low stringency (55°C, 5 X SSC). Five mRNA species, ranging in size from 0.8 to 5 kb, were detected (Fig. 16). This low stringency washing condition allowed us to detect three new mRNA species that were not seen at higher stringency [14]. These mRNAs may encode the different proteins recognized by the anti-Wcs120 antibody. We have recently isolated several anti-Wcs120 positive clones. One of these has a cDNA insert size of 4.7 kb and encodes the 200 kDa protein [30]. We thus believe that the proteins revealed by the anti-Wcs120 antibody are encoded by different genes.

Accumulation kinetics of freezing tolerance markers (FTMs) during cold-acclimation

The anti-Wcs120 antibody was reacted against soluble proteins isolated from cold-acclimated seedlings. Equal amounts of proteins separated by gel electrophoresis were used in assays in any given cultivar. Since the cultivar Glenlea is relatively freezing sensitive, it is likely to synthesize less FTMs

and, therefore, 50% more proteins were loaded to increase the chance of immunodetection (compare Fig. 17A and 17C). Results in Fig. 17B show that proteins from non-acclimated seedlings react very weakly to this antibody. When the seedlings were transferred to 4°C, FTMs accumulated rapidly. A large amount of these proteins was seen after 6 days of cold acclimation in both Fredrick (more tolerant) and Glenlea (less tolerant) cultivars. The accumulation of these FTMs was more pronounced and began earlier in Fredrick (Fig. 17B) and the level was maintained or continued to increase slightly during the 36 days of cold treatment. On the other hand, these FTMs had reached their maximum levels in Glenlea after 15 days of cold acclimation and they then declined despite the seedlings being maintained at 4°C. When the plants were returned to normal growth conditions, the FMTs declined rapidly and reached near control level after 6 days of deacclimation for Fredrick and after only 1 day for Glenlea. These results indicate that the accumulation of FTMs is correlated with the capacity of each genotype to develop freezing tolerance during the cold acclimation period.

Expression of FTMs in different tissues and during other stresses and ABA treatments

On comparing the relative abundances of these proteins in leaves, crown and roots, we concluded that FTMs accumulate to higher levels in those parts of the plant with the highest freezing tolerance. Figure 18A shows that the leaves and meristematic crown (more tolerant) contain more FTMs than the basal region of the crown and roots (less tolerant).

FTMs are specifically induced by low temperature (Fig. 18B). These proteins are slightly induced by water stress and ABA, while they are not induced by heat shock or salt stress. Interestingly, the antibodies recognized proteins of lower molecular weight (14 to 21 kDa) that are strongly induced by water stress and ABA. These proteins presumably belong to the RAB and/or dehydrin families since proteins of that molecular weight range have been shown to be induced during water stress and ABA treatments [43, 47, 5]. Furthermore, the Wcs120 sequence shares homology with these two protein families through two repeated motifs [14]. In order to verify that dehydrins are recognized by our antibody, barley seedlings were subjected to water stress and the soluble proteins were extracted and reacted with the anti-Wcs120 antibody. The results in Fig 18B show that the low molecular weight proteins strongly induced during water stress were not detected in cold-treated

barley. Moreover, a protein of ca. 72 kDa was strongly induced by cold treatment while it was only slightly induced by water stress. This indicates that proteins strongly induced by cold treatment are induced either poorly or not at all by water stress.

The role of ABA in the induction of freezing tolerance was investigated in two wheat (T. aestivum L.) cultivars, Glenlea (spring var) and Fredrick (winter var). Exogenous application of ABA (5 X 10^{-5}M for 5 days at 24°C) increased the freezing tolerance of intact plants by only 3°C (LT_{50}) in both cultivars. Maximal freezing tolerance (LT_{50} of -9°C for Glenlea and -17°C for Fredrick) could only be obtained with a low temperature treatment (6/2; day/night) for 40 days. These results show that exogenously applied ABA cannot substitute for low temperature requirement to induce freezing tolerance in intact wheat plants. Furthermore, there was no increase in the endogenous ABA level of wheat plants at any time during low temperature acclimation, suggesting the absence of an essential role for ABA in the development of freezing tolerance of intact plants. On the other hand, ABA application (5 X 10^{-5} M for 5 days at 24°C) to embryogenic wheat calli resulted in an increase of freezing tolerance similar to that achieved by low temperature. However, as in intact

plants, there was no increase in the endogenous ABA level during low temperature acclimation of calli. These results indicate that the induction of freezing tolerance by low temperature is not associated with an increase in ABA content. Using the anti-Wsc120 antibody specific to a protein family associated with the development of freezing tolerance, we demonstrated that the induction of freezing tolerance by ABA in embryogenic wheat calli was correlated with the accumulation of a new 32KDa protein. This protein is specifically induced by ABA but shares a common antigenicity with those induced by low temperature. These results suggest that ABA induces freezing tolerance in wheat calli via a regulatory mechanism different from that of low temperature.

Species specificity of FTMs

In order to determine whether proteins similar to Wcs120 accumulate during cold acclimation in other species, we examined a number of tolerant and less tolerant cereals as well as four tolerant dicot species. Figure 19 shows that proteins from tolerant cereals (lanes 1-3) reacted most strongly with the anti-Wcs120 antibody. Less tolerant cereals (lanes 4-6) reacted less strongly. It appears that the correlation between

LT_{50} and FTMs may not be perfect for all graminaceous species, as shown in the case of oat (lane 5). The antibody did not recognize any protein in the sensitive cereal varieties (lanes 7 and 8) or within the tolerant dicotyledonous group (lanes 9-12). This suggests that these FTMs are not associated with cold acclimation in these species or that the proteins have evolved in a way that does not allow cross-reactivity with the antibody.

The Wcs120 cDNA was shown to hybridize with at least five different mRNA species, which have the potential to encode all the proteins recognized by the anti-Wcs120 antibody. The accumulation kinetics of these coordinately expressed proteins are positively correlated with the time of development of the degree of freezing tolerance. Hence, we refer to this protein family as Freezing Tolerance Markers [FTMs]. The winter wheat variety Fredrick accumulates more of these FTMs and continues accumulating them throughout the cold acclimation period. In contrast, the spring variety Glenlea accumulates less FTMs and their level starts to decline early during the acclimation period correlating with the sharp reduction in mRNA seen in our previous study [14]. Moreover, FTMs accumulated in greater amounts in tissues with higher capacity to develop freezing tolerance. This observation is consistent with

our earlier observation, which showed a preferential synthesis of the 200 kDa protein (a member of this protein family) at the shoot level compared with the roots [31].

We have also demonstrated that the antibody recognizes several proteins in other cold-acclimated cereals. The amount of cross-reactive material was much higher in the freezing-tolerant species. These results confirm the positive correlation between the induction of these proteins and the capacity of plant or tissues to develop freezing tolerance. Furthermore, the accumulation of this protein family did not appear to be associated with the plant developmental stage since it was induced at low temperature in calli, sprouts, and germinating seedlings at different growth stages (not shown). The expression of FTMs was induced to the same level in dark or light-grown seedlings, suggesting that this is light independent.

No cross-reactivity could be found in freezing-tolerant dicotyledonous species suggesting that freezing tolerance in monocots and dicots involves different proteins. However, these proteins may have similar properties (such as boiling stability or high hydrophilicity). The presence of proteins of variable molecular weight having a similar antigenicity is novel

and suggests that their function is determined through common small repeated elements within their structure. Such repeats do exist within the 50 kDa [14] and 200 kDa proteins [30]. Similar motifs have been found in the dehydrin [5, 1] and RAB [43, 47] families. These results suggest that the number of repeats within the protein molecule and the amount of these proteins synthesized are important factors in the acquisition of freezing and drought tolerance. However, the induction of proteins of different sizes during cold and drought stress (in both wheat and barley) suggests that they probably have distinct functions with some complementarity.

Some of the FTMs induced by cold are also induced slightly by water stress and ABA but they are not induced by salt stress or heat shock. Evidence in the literature indicates that ABA and drought can increase freezing tolerance [6, 10, 39]. However, in the case of the intact wheat plant, the freezing tolerance conferred by ABA is -8°C (-4°C for the non-acclimated control) compared with -16°C after cold acclimation [10, 31]. This may be due to the partial induction of FMTs and other cold-regulated genes necessary for the acquisition of freezing tolerance. This assumption requires a detailed study to determine the exact role that ABA and

drought may play during cold-acclimation. It is however interesting to observe that small molecular weight proteins recognized by the anti-Wcs120 antibodies are present in high concentration during water stress and ABA treatment. This suggests that low temperature induction of freezing tolerance involves pathways distinct from that induced by ABA and water stress. A similar conclusion has been reached in studies, with Arabidopsis thaliana [28].

The characterization of FTMs should help us understand their role in the acquisition of freezing tolerance. However, the antibody represents a very important and easily accessible tool to identify cereal cultivars with a superior capacity for cold acclimation. This potential can be assessed rapidly with proteins extracted from as little as 50 mg of plant tissues. This provides breeders with a simple and economic method of selection for potential freezing tolerance of new cereal crops.

Understanding the molecular genetic bases of cold acclimation in wheat requires the identification of genes involved in this complex mechanism. Towards this goal, we have identified and characterized several cDNA clones [14, 15, 30]. The cDNA clones Wcs19, Wcor410 and Wcs120 described here represent novel genes which are

regulated by low temperature. These clones were identified by screening a cDNA library with a subtracted probe to reduce the high signal of abundant mRNAs and thus facilitate the isolation of new cDNAs. Northern analyses have shown that the mRNAs accumulate only when the plants are exposed to low temperature. After 24 hours of exposure, the three genotypes accumulated mRNAs to nearly their maximal levels indicating that the accumulation is very rapid. The mRNA levels remained constant thereafter in the two winter genotypes while it declined in the less tolerant spring genotype after 36 days of low temperature exposure. This result suggests that a constant level of mRNAs may be necessary to allow the accumulation of a sufficient amount of proteins required for the development of FT. Southern analysis has not shown any differences in restriction patterns between the most tolerant and less tolerant genotypes. One might infer that the promoter structure is different and could not be detected by simple restriction analysis. This possibility is not likely since all genotypes accumulate the cold-induced mRNAs to similar levels at early stages of cold acclimation. We do not yet understand the mechanism underlying this differential expression.

Our results indicate these mRNAs are expressed specifically in tolerant gramineae species. There was no detectable expression in sensitive gramineae such as rice or in any of the tolerant dicot species examined. However, this does not exclude the possibility that proteins with a similar function are present in dicots. Moreover, genes Wcs19 and Wcs120 are not induced by ABA, drought, heat shock, salt, wounding, or anaerobic stresses indicating that the gene is induced specifically by low temperature. Sequence comparison of Wcs19 did not reveal any homology with other published genes or with other genes cloned in our laboratory. Furthermore, we have not found any of the repeated sequences observed in RAB [26], dehydrins [5], or LEA [1] while such repeated sequences were found in Wcs120. This suggests that Wcs19 plays a distinct role in cold acclimation. Several structural properties of this protein are similar to those found in transcription factors. The C-terminal half of the protein contains all the acidic residues with a net charge of -7. In addition, an alpha helical structure is predicted for the last 91 amino acids. It has been suggested that an alpha helical structure might be an essential element of the activating region of several transcription factors [32]. Furthermore, the acidic character of the

activating region is an important feature which allows transcription factors to interact with RNA polymerases and increase transcription rates [32]. It has been shown that the overall RNA polymerase activity increases markedly during cold acclimation [38]. This could contribute to increase the expression of several genes important for the development of FT.

Another important feature of transcription factors is the presence of a positively charged domain. This domain is essential for the interaction with DNA. In Wcs19, the N-terminal half is rich in proline (14 %) and contains basic residues (lysine and arginine) with a net charge of +10. Those characteristics are found in transcription factors such as CPRF-1 [44], HBP1-a [41], and CTF/NF-1 [23]. However, detailed experiments are required to confirm this hypothesis.

Our results indicate that Wcs19 expression is leaf-specific and not directly associated with FT in other plant tissues or callus cultures. It seems that its expression is dependent on leaf tissue organization and on the plant capacity to acclimate at low temperature. The role of leaf during cold acclimation is to provide the energy required for the development of FT. Photosynthesis is responsible for the production of this energy and it has been shown that tolerant

cultivars have a higher photosynthetic capacity than the spring ones [2, 29]. This modulation of the photosynthetic apparatus by the tolerant plants to optimize growth at suboptimal conditions is correlated with an increased resistance to photoinhibition [29]. The close correlation of Wcs19 gene expression with the capacity of leaves to develop FT suggests that this gene may play a role in this process.

The specific expression in the leaf indicates that cell or callus cultures mimic only in part the response of intact plants to low temperature stress. Our results emphasize that the leaf tissue response could be very important to the survival of the whole plant. It appears from these observations, and that of others [3, 21, 46], that we must distinguish between cellular responses to low temperature which may be ubiquitous to all cells from the tissue-specific reactions. This is an important consideration when one wants to improve FT at the whole plant level.

On the other hand, the presence of repeated sequences in Wcs120 in common with RAB and dehydrins indicate a common feature or function. A high proportion of hydrophilic residues is present in all three protein families, as is the case of LEA proteins present in maturing embryos [1], and for cold-induced

proteins found in other plants [3, 9, 17], in insects [49], and in E. coli [12]. The absence of hydrophobic regions indicated that these proteins are probably not membrane-bound.

It has been reported for wheat and Arabidopsis that several polypeptides induced are accumulated during cold treatment remain soluble during boiling in aqueous solution [7, 20]. This property was previously observed for LEA proteins and dehydrins [5, 16]. The large number of hydrophilic residues probably confers a very flexible backbone and this is likely responsible for boiling stability of these proteins, including the 50-kD protein identified in our work (Wcs120), because they would not have to renature after boiling. The high Gly content (26%) of the 50-kD protein may confer a high flexibility and mobility to the protein as found in several Gly-rich proteins such as elastin. The small size of the Gly molecule and its short side chain gives it a unique function in the structure of several proteins. It facilitates the formation of intramolecular hydrogen bonding and thus gives the protein a random coil conformation. This property allows the protein to stretch, bend, and expand in all directions, a property that could be useful to protect cellular structures against freezing or severe

dehydration. Physicochemical characterization of this protein should help to verify this assumption. The significance of these properties is still unclear but the high hydrophilicity may also be important in hydrogen bonding to the lattice of nascent ice crystals, thus modifying the structure or propagation of ice crystals, which may reduce intracellular freezing damage during winter. The high hydrophilicity of these proteins may also be important in trapping enough water inside the cell to prevent local dehydration that may occur during freezing or water stress [13, 40].

Drought stress was shown to increase the freezing tolerance [13]. This suggests that some features must be common between the proteins induced during these different stresses.

The absence of the serine-rich repeat and the specific induction of Wcs120 mRNA early during cold acclimation, before any increase in osmotic pressure, cell dehydration, or ABA content occurs (data not shown), suggested that the Wcs120 gene was regulated differently from the RAB and dehydrin families. The molecular mass of the Wcs120 protein is much higher than the known RAB and dehydrin protein masses. The striking recurrence of the common repeats in Wcs120, RAB, and dehydrin proteins suggests that survival at low

temperature and during water stress requires large amounts of these unusual proteins.

Protective effect of purified proteins on the denaturation of glutathione reductase by different treatments.

The inventors have evaluated the protective effect of the purified proteins Wcs120 and Wcor410 on the denaturation of glutathione reductase by different treatments.

Denaturation of glutathione reductase (Type III, Sigma) was performed by incubating 14 mU of enzyme in 50 µl of 100 mM KPO_4 buffer pH 7.5. The starting activity was measured and this value was set at 100% of activity (control). The different preparations were incubated either alone (GR alone) or in the presence of the indicated amount of purified proteins. Bovine serum albumin was purchased from Pharmacia (BSA, DNase free 10 mg/ml); WCS120 was expressed and purified as described in this application; WCOR410 was expressed by subcloning the coding region into the vector pET22b. The protein was purified using the His*Bind™ buffer kit protocol described by the company (Novagen).

The different denaturation treatments were as follows: Heat denaturation was achieved by incubating

the above enzyme preparations at 60°C for 10 min; Desiccation was achieved by evaporating the above enzyme preparations to dryness under vacuum (45 min). The enzyme was reconstituted to the original volume before measuring the remaining activity; Cold denaturation was achieved by incubating the above enzyme preparations at -20°C for 24 hours.

The remaining enzyme activity was determined by incubating 15 µl (4.2 mU) of the different enzyme preparations and the results expressed as a percent of the control value. The reaction (200 µl final volume) was initiated by adding the enzyme to the following: 100 µl of 100 mM KPO_4 buffer pH 7.5, 50 µl of 3 mM 5,5'-dithiobis-(2-nitrobenzoic acid) prepared in 10 mM KPO_4 pH 7.5, 10 µl of 20 mM oxidized glutathione, 10 µl of 2 mM NADPH, and 15 µl of H_2O. The activity was measured spectrophotometrically by following the absorbance at 412 nm.

Results represented in Table I, show that the glutathione reductase is sensitive to the different treatments and can be protected by the addition of purified proteins. BSA is used as a reference since it is a well known protein used to increase enzyme stability under a variety of conditions. Used at 100 and 10 µg/ml, the different proteins appear to give

equivalent protection against heat denaturation, desiccation, or cold denaturation. However, used at 1 µg/ml, both WCS120 and WCOR410 are more efficient at protecting against cold denaturation. These in vitro tests suggest that the isolated proteins may play a role as general protectants for enzymes or other cellular structures in vivo. We are now performing transformation experiments to express these and other proteins in transgenic plants in order to evaluate the degree of protection conferred by these proteins against heat, cold and desiccation stresses in vivo.

TABLE I

Treatments		60°C	Desiccation	-20°C
control		100%	100%	100%
GR alone		24	21	4
GR + BSA	100 µg/ml	66	75	-
	10	57	57	71
	1	39	24	13

GR + WCS120	100	81	79	-
	10	80	56	80
	1	63	22	40
GR + WCOR410	100	-	81	-
	10	-	69	68
	1	-	23	42

The genes disclosed in this application are intended to be used in the construction of expression vectors which will produce the encoded proteins, these proteins conferring freezing resistance to host cells, bacteria or plants tissues, or these proteins when extracted being usable as anti-freezing agents.

The production of economic plant species which would be freezing-resistant is particularly envisaged.

REFERENCES

1. Baker J, Steele C, Dure III, L: Sequence and characterization of 6 lea proteins and their genes from cotton. Plant Mol Biol 11: 277-291 (1988).

2. Cadieux C, Sarhan F, Perras M: Osmotic adjustment and photosynthetic electron transport response to cold hardening in winter and spring wheat. Plant Physiol Biochem 26: 313-322 (1988).

3. Cattivelli L, Bartels D: Molecular cloning and characterization of cold-regulated genes in barley. Plant Physiol 93: 1504-1510 (1990).

4. Chevrier N, Qureshi JA, Hucl P, Kartha KK: Heritability of in vitro regeneration in wheat (Triticum aestivum L.). Can J Plant Sci 70: 547-550 (1990).

5. Close TJ, Kortt AA, Chandler PM: A cDNA-based comparison of dehydrationinduced proteins (dehydrins) in barley and corn. Plant Mol Biol 13: 95-108 (1989).

6. Cox W, Levitt J: Interrelation between environmental factors and freezing resistance of cabbage leaves. Plant Physiol 57:553-555 (1976).

7. Danyluk J, Rassart É, Sarhan F: Gene expression during cold and heat shock in wheat. Biochem Cell Biol 69: 383-391 (1991).

8. Danyluk J, Sarhan, F: Differential mRNA transcription during the induction of freezing tolerance in spring and winter wheat. Plant Cell Physiol 31: 609-619 (1990).

9. Dunn MA, Hughes MA, Pearce RS, Jack PL: Molecular characterization of a barley gene induced by cold treatment. J Exp Bot 41: 1405-1413 (1990).

10. Gagné Y: Rôle de l'acide absicissique lors de l'acclimatation au froid chez le blé MSc. Thesis, Université du Québec à Montréal (1991).

11. Garnier J, Osguthorpe DJ, Robson B: Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J Mol Biol 120: 97-120 (1978).

12. Goldstein J, Pollitt NS, Inouyé M: Major cold shock protein of Escherichia coli. Proc Natl Acad Sci USA 87: 283-287 (1990).

13. Guy CL: Cold acclimation and freezing stress tolerance: role of protein metabolism. Ann Rev Plant Physiol Plant Mol Biol 41: 187-223 (1990).

14. Houde M, Danyluk J, Laliberté JF, Rassart É, Dhindsa RS, Sarhan F: Cloning, Characterization, and expression of a cDNA encoding a 50-kilodalton protein specifically induced by cold acclimation in wheat. Plant Physiol 99: 1381-1387 (1992).

15. Houde M, Dhindsa RS, Sarhan F: A molecular marker to select for freezing tolerance in gramineae. Mol Gen Genet 234: 43-48 (1992).

16. Jacobsen JV, Shaw DC: Heat stable proteins and abscisic acid action in barley aleurone cells. Plant Physiol 91: 1520-1526 (1989).

17. Kurkela S, Franck M: Cloning and characterization of a cold- and ABA-inducible Arabidopsis gene. Plant Mol Biol 15: 137-144 (1990).

18. Kyte J, Doolittle RF: A simple method for displaying the hydrophobic character of a protein. J Mol Biol 157:105-132 (1982).

19. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685 (1970).

20. Lin C, Guo WW, Everson E, Thomashow MF: Cold acclimation in Arabidopsis and wheat. Plant Physiol 94: 1078-1083 (1990).

21. Lin C, Thomashow M: DNA sequence analysis of a complementary DNA for coldregulated Arabidopsis cor15 and characterization of the cor15 polypeptide. Plant Physiol 99: 519-525 (1992).

22. Luo M, Liu JH, Mohapatra S, Hill RD, Mohapatra SS: Characterization of a gene family encoding abscisic acid- and environmental stress-inducible proteins of alfalfa. J Biol Chem 267:15367-15374 (1992).

23. Mermod N, O'neill EA, Kelly TJ, Tijian, R: The proline-rich transcriptional activator of CTF/NF-1 is distinct from the replication and DNA binding domain. Cell 58: 741753 (1989)

24. Mohapatra SS, Poole RJ, Dhindsa RS: Abscisic acid-regulated gene expression in relation to freezing tolerance in Alfalfa. Plant Physiol 87: 468-473 (1988).

25. Mohapatra SS, Wolfraim L, Poole RJ, Dhindsa RS: Molecular cloning and relation to freezing tolerance of cold-acclimation-specific genes of Alfalfa. Plant Physiol 89: 375-380 (1989)

26. Mundy J, Chua NH: Abscisic acid and water stress induce the expression of a novel rice gene. EMBO J 7: 2279-2286 (1988).

27. Murashige T, Skoog F: A reversed medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol 15: 473-497 (1962).

28. Nordin K, Heino P, Palva ET: Separate signal pathways regulate the expression of a low-temperature-induced gene in Arabidopsis thaliana (L.) Heynh. Plant Mol Biol 16: 1061-1071 (1991).

29. Oquist G, Vaughan MH, Huner NPA: Low-temperature effects on photosynthesis and correlation with

freezing tolerance in spring and winter cultivars of wheat and rye. Plant Physiol 101: 245-250 (1993).

30. Ouellet F, Houde M, Sarhan, F: Cloning of the 200 kDa protein induced by cold acclimation in wheat. Plant Cell Physiol 34: 59-65 (1993).

31. Perras M, Sarhan F: Synthesis of freezing tolerance proteins in leaves, crown and roots during cold acclimation of wheat. Plant Physiol 89: 577-585 (1989).

32. Ptashne M: How eukaryotic transcriptional activators work. Nature 335: 683-689(1988).

33. Rocher-Chambonet C, Berreur P, Houde M, Tiveron MC, Lepesant JA, Brégégère F: Cloning and partial characterization of the xanthine dehydrogenase gene of Calliphora vicina, a distant relative of Drosophila melanogaster. Gene 59: 201-212 (1987).

34. Rogers SO, Bendich AJ: Extraction of DNA from plant tissues. Plant Mol Biol Manual A6: 1-10 (1988).

35. Rosen KM, Villa-Komaroff L: An alternative method for the visualization of RNA in formaldehyde agarose gels. Focus 12: 23-24 (1990).

36. Sambrook J, Fritsch EF, Maniatis T: Molecular cloning: A laboratory manual, 2nd ed. Cold Spring Harbor, New York (1989).

37. Sanger F, Nicklen S, Coulson AR: DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463-5467 (1977).

38. Sarhan F, Chevrier N: Regulation of RNA synthesis by DNA-dependent RNA polymerase and RNases during cold acclimation in winter and spring wheat. Plant Physiol 78: 250-255 (1985).

39. Siminovitch D, Cloutier Y: Twenty-four hour induction of freezing and drought tolerance in plumules of winter rye seedlings by desiccation stress at room temperature and in the dark. Plant Physiol 69: 250-255 (1982).

40. Steponkus PL, Lynch DV: Freeze/thaw-induced destabilization of the plasma membrane and the

effects of cold-acclimation. J Bioenerg Biomembr 21: 21-41 (1989).

41. Tabata T, Takase H, Takayama S, Mikami K, Nakatsuka A, Kawata T, Nakayama T, Iwabuchi M: A protein that binds to a cis-acting element of wheat histone gene has a leucine zipper motif. Science 245: 965-967 (1989).

42. Thomashow MF: Molecular genetics of cold acclimation in higher plants. Adv Genet 28: 99-31 (1990).

43. Vilardell J. Goday A. Freire MA, Torrent M, Martinez MC, Torné JM, Pagès M: Gene sequence, developmental expression, and protein phosphorylation of Rab-17 in maize. Plant Mol Biol 14: 423-432 (1990).

44. Weisshaar B, Armstrong GA, Block A, Oswaldo CS, Hahlbrock K: Light inducible and constitutively expressed DNA-binding proteins recognizing a plant promoter element with functional relevance in light responsiveness. EMBO J 10: 1777-1786 (1991).

45. Weretilnyk EA, Hanson AD: Molecular cloning of a plant betaine-aldehyde dehydrogenase, an enzyme implicated in adaptation to salinity and drought. Proc Natl Acad Sci USA 87: 2745-2749 (1990).

46. Weretilnyk E, Winson O, White TC, lu B, Singh J: Characterization of three related low-temperature-regulated cDNAs from winter Brassica Napus. Plant Physiol 101: 171-177 (1993).

47. Yamaguchi-Shinozaki K, Mundy J, Chua N-H: Four tightly linked rab genes are differentially expressed in rice. Plant Mol Biol 14: 29-39 (1989).

48. Yanish-Perron C, Vieira J, Messing J: Improved M13 phage cloning vectors and host strains: nucleotide sequences of M13mp18 and pUC19 vectors. Gene 33: 103-119 (1985).

49. Zachariassen KE: Physiology of cold tolerance in insects. Physiol Rev 65: 799-832 (1985).

50. Guo W, Ward R W, Thomashow M F: Characterization of a Cold-Regulated Wheat Gene Related to Arabidopsis cor47. Plant Physiol. 915-922 (1992).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANTS: SARHAN, Fathey
HOUDE, Mario
LALIBERTÉ, Jean-François

(ii) TITLE OF THE INVENTION: DNA MOLECULES ENCODING FREEZING TOLERANCE PROTEINS IN GRAMINEAE

(iii) NUMBER OF SEQUENCES: 6

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5 inch, 720 kb diskette
(B) COMPUTER: IBM PS/2, Model 30
(C) OPERATING SYSTEM: PC-DOS 3.30
(D) SOFTWARE: WordPerfect 5.1

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE: August 16, 1993
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Ms. Mary J. Wilson
(B) REGISTRATION NUMBER:
(C) REFERENCE/DOCKET NUMBER:

(ix) TELECOMMUNICATION INFORMATION:

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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 943
(B) TYPE: Nucleic acid
(C) STRANDENESS: Double
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA to mRNA

(vi) ORIGINAL SOURCE: Triticum Aestivum L.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1

```
TTTTTTTTTG CGACCAAAAT GAACAAGTAA ATTTACTCCC TCACAAGCAT      50
ATGCAAATAT ATTCCACCAA GAATATTAGT CGGTCCTCGC TATCAACCAC     100
ATCTAAAACC ATGTCAACGA ATGGAAACAA CACCACCTTA AAAGTATCCA     150
CACGAGAAGG CTCCTTATAT TTGTATTAAC AGAAGAGCAA AAAGATATAG     200
CTGTATGATT TCAGCGATCC AAATCCGCAT GGTGCAGCGA TGCGCAAGAC     250
TACCATTTCC AATCGGCACA CATCCTGTCT CCTTCCACAA CCTACCCTAC     300
CCACCCATCC ATCAGCAGTT TTTCTATCGA CCAATGGCTT CTTCTTCCGT     350
GCTGCTCGGA GCCTCGGCCA CGGCCGCGCT CACCGGCACC CCGGCAGGCA     400
AGGCCCTTCC CCGGCCTTGC TTCCTCGCCG CTCGCCCGCG CACCGTGAGC     450
GGTGGCCGTC TCTGCCTGCA GAACGCTCCA AGGGCGACTC CGGCGTACAA     500
CGACGCTGCG GATGCCACCG ACAAGGCCAT CGACGGCGTG AAGGGGGTGG     550
CCGACGAGTT GAAGAAGGGC GTGGCGGAGG CTGCGGAGGC CGTCTCGGGC     600
AACACCGAGA AGGCCGCGGA GGAAGCCGGC AAGGGCGCGA GCGAGGTGGA     650
CGCGAAGGCC AAGGACTTCG GCGAGCAGGC GAAGAAGGCG ACGGAGGAGG     700
CGTGGGACGG CGCCAAGGAC GCCGCACAGG GCATCACGGA CAAAGTCGCC     750
GCCGCGGCCA AAAAGGAAGC TAGCTAAGCT AACACTACGT TGACTAGTCC     800
GATCTGTATC GCTCAATTCA TTTTCCATTG TAAGGAATGC ATATACGTAT     850
TTCGGTACAA GAGATAAGAT AGCTGTATTT ATTTTCTGTG ATATAGGATT     900
ACCGCACTGT TAATGTCAAA CGCAATAAAG AAAATGATTT TTY            943
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 190
(B) TYPE: Amino acid
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Protein

(vi) ORIGINAL SOURCE: Triticum Aestivum L.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2

```
Met Ile Ser Ala Ile Gln Ile Arg Met Val
                5                  10
Gln Arg Cys Ala Arg Leu Pro Phe Pro Ile
               15                  20
Gly Thr His Pro Val Ser Phe His Asn Leu
               25                  30
Pro Tyr Pro Pro Thr His Gln Gln Phe Phe
               35                  40
Tyr Arg Pro Met Ala Ser Ser Ser Val Leu
               45                  50
Leu Gly Ala Ser Ala Thr Ala Ala Leu Thr
               55                  60
Gly Thr Pro Ala Gly Lys Ala Leu Pro Arg
               65                  70
Pro Cys Phe Leu Ala Ala Arg Pro Arg Thr
```

```
                    75                  80
Val Ser Gly Gly Arg Leu Cys Leu Gln Asn
                    85                  90
Ala Pro Arg Ala Thr Pro Ala Tyr Asn Asp
                    95                 100
Ala Ala Asp Ala Thr Asp Lys Ala Ile Asp
                   105                 110
Gly Val Lys Gly Val Ala Asp Glu Leu Lys
                   115                 120
Lys Gly Val Ala Glu Ala Ala Glu Ala Val
                   125                 130
Ser Gly Asn Thr Glu Lys Ala Ala Glu Glu
                   135                 140
Ala Gly Lys Gly Ala Ser Glu Val Asp Ala
                   145                 150
Lys Ala Lys Asp Phe Gly Glu Gln Ala Lys
                   155                 160
Lys Ala Thr Glu Glu Ala Trp Asp Gly Ala
                   165                 170
Lys Asp Ala Ala Gln Gly Ile Thr Asp Lys
                   175                 180
Val Ala Ala Ala Ala Lys Lys Glu Ala Ser
                   185                 190
```

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1522
(B) TYPE: Nucleic acid
(C) STRANDENESS: Double
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA to mRNA

(vi) ORIGINAL SOURCE: Triticum Aestivum L.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3

```
CGAGTGAGGA GCTCAGCGCA AGATGGAGAA CCAGGCACAC ATCGCCGGCG      50
AGAAGAAGGG CATCATGGAG AAGATCAAGG AGAAGCTCCC CGGCGGCCAC     100
GGCGACCACA AGGAGACCGC TGGTACCCAC GGGCACCCCG GCACGGCGAC     150
GCATGGTGCC CCGGCCACTG GTGGTGCCTA CGGGCAGCAG GGTCACGCTG     200
GAACCACCGG CACGGGGTTG CATGGCGCCC ACGCCGGCGA GAAGAAGGGC     250
GTCATGGAGA ACATCAAGGA CAAGCTCCCT GGTGGCCACC AGGACCACCA     300
GCAGACTGGT GGTACCTATG GGCAGCAGGG ACACACCGGC ACGGCGACGC     350
ATGGCACCCC GGCGACCGGT GGCACCTATG GGCAGCAGGG ACATACCGGC     400
ACAGCGACGC ATGGCACCCC GGCGACCGGT GGCACCTATG GGGAGCAGGG     450
ACACACCGGA GTGACTGGCA CGGGGACGCA CGGCACCGGC GAGAAGAAGG     500
CGGTGACCAC GAACATCAAG GAGAAGCTCC CTGGTGGCCA CGGCACCGGC     550
CAGCAGACCG GTGGTACCTA CGGGCAGCAG GGACACACCG GCACGGCGAC     600
GCATGGCACC CCGGCCGGGG GCGGCACCTA TGAGCAGCAC GGACACACCG     650
GGATGACCGG CACAGGGACA CACGGCACTG GCGAGAAGAA AGGCGTCATG     700
GAGAACATCA AGGACAAGCT CCCTGGTGGC CACGGAGATC ACCAGCAGAC     750
CGGTGGCACC TACGGGCAGC AGGGACACAC CGGCACGGCG ACACAGGGCA     800
CCCCGGCCGG CGGCGGCACC TATGAGCAGC ATGGACACAC CGGGATGACC     850
```

```
GGCGCGGGGA CACACAGCAC TGGCGAGAAG AAGGGCGTCA TGGAGAACAT      900
CAAGGAAAAG CTCCCTGGTG GCCACAGTGA CCACCAGCAG ACCGGTGGAG      950
CCTACGGGCA GCAGGGACAC ACCGGCACGC GACACATGGC ACCCCTGCCG     1000
GCGGGCACCT ACGGGCAGCA TGGACACGCT GGAGTGATCG GCACGGAGAC     1050
GCATGGCACC ACGGCCACCG GCGGCACCCA TGGGCAGCAC GGACACACCG     1100
GAACGACTGG CACTGGGACA CACGGCTCCG ACGGGATCGG CGAGAAGAAG     1150
AGCCTCATGG ACAAGATCAA GGATAAGCTG CCTGGACAGC ACTGAGCCCG     1200
GTCTGCCCGC GGCCGCTACC CTTGCAGAAT AATAACCCCA CCGTGTATAA     1250
GTTAATTGAG TCTAGTTCAC CTAGCTCACT TGGTCGTTGG AGGAGAGAAT     1300
GTATTATGTA TCTTGGTTTA AGTTTTCACG GACAACAGTG TGTTCACAGT     1350
TTTCTTCTGT TTACACTCTG TAGTGCAAAT TCGTTTAAGT TTTCACGGAC     1400
AACAGTGTGT TCACAGTTTT CTTCTGTTTA CACTCTGTAG TGCAAATTTC     1450
GTTTTTGTTC TTTTTTTTTT TGTCCATCTT ATCCAAGAGA CAGACGCAGC     1500
GAAAAAAAAA AAAAAAAAAA AA                                   1522
```

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 390
(B) TYPE: Amino acid
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Protein

(vi) ORIGINAL SOURCE: Triticum Aestivum L.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4

```
Met Glu Asn Gln Ala His Ile Ala Gly Glu
                  5                  10
Lys Lys Gly Ile Met Glu Lys Ile Lys Glu
                 15                  20
Lys Leu Pro Gly Gly His Gly Asp His Lys
                 25                  30
Glu Thr Ala Gly Thr His Gly His Pro Gly
                 35                  40
Thr Ala Thr His Gly Ala Pro Ala Thr Gly
                 45                  50
Gly Ala Tyr Gly Gln Gln Gly His Ala Gly
                 55                  60
Thr Thr Gly Thr Gly Leu His Gly Ala His
                 65                  70
Ala Gly Glu Lys Lys Gly Val Met Glu Asn
                 75                  80
Ile Lys Asp Lys Leu Pro Gly Gly His Gln
                 85                  90
Asp His Gln Gln Thr Gly Gly Thr Tyr Gly
                 95                 100
Gln Gln Gly Thr His Gly Thr Ala Thr His
                105                 110
Gly Thr Pro Ala Thr Gly Gly Thr Tyr Gly
                115                 120
Gln Gln Gly His Thr Gly Thr Ala Thr His
                125                 130
Gly Thr Pro Ala Thr Gly Gly Thr Tyr Gly
```

```
                    135                                 140
Glu Gln Gly His Thr Gly Val Thr Gly Thr
                    145                                 150
Gly Thr His Gly Thr Gly Glu Lys Lys Gly
                    155                                 160
Val Met Glu Asn Ile Lys Glu Lys Leu Pro
                    165                                 170
Gly Gly His Gly Asp His Gln Gln Thr Gly
                    175                                 180
Gly Thr Tyr Gly Gln Gln Gly His Thr Gly
                    185                                 190
Thr Ala Thr His Gly Thr Pro Ala Gly Gly
                    195                                 200
Gly Thr Tyr Glu Gln His Gly His Thr Gly
                    205                                 210
Met Thr Gly Thr Gly Thr His Gly Thr Gly
                    215                                 220
Glu Lys Lys Gly Val Met Glu Asn Ile Lys
                    225                                 230
Asp Lys Leu Pro Gly Gly His Gly Asp His
                    235                                 240
Gln Gln Thr Gly Gly Thr Tyr Gly Gln Gln
                    245                                 250
Gly His Thr Gly Thr Ala Thr Gln Gly Thr
                    255                                 260
Pro Ala Gly Gly Gly Thr Tyr Glu Gln His
                    265                                 270
Gly His Thr Gly Met Thr Gly Ala Gly Thr
                    275                                 280
His Ser Thr Gly Glu Lys Lys Gly Val Met
                    285                                 290
Glu Asn Ile Lys Glu Lys Leu Pro Gly Gly
                    295                                 300
His Ser Asp His Gln Gln Thr Gly Gly Ala
                    305                                 310
Tyr Gly Gln Gln Gly His Thr Gly Thr Arg
                    315                                 320
His Met Ala Pro Leu Pro Ala Gly Thr Tyr
                    325                                 330
Gly Gln His Gly His Ala Gly Val Ile Gly
                    335                                 340
Thr Glu Thr His Gly Thr Thr Ala Thr Gly
                    345                                 350
Gly Thr His Gly Gln His Gly His Thr Gly
                    355                                 360
Thr Thr Gly Thr Gly Thr His Gly Ser Asp
                    365                                 370
Gly Ile Gly Glu Lys Lys Ser Leu Met Asp
                    375                                 380
Lys Ile Lys Asp Lys Leu Pro Gly Gln His
                    385                                 390
```

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1136
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA to mRNA

(vi) ORIGINAL SOURCE: Triticum Aestivum L.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5

```
AAAAGCCACA AGCCAAGAAC CAATACTTGA TCTGTTGTTT CCTTTAGCTC      50
CCGGAAGACT TTTAGCTGCA CCGATCGATC TCGATCATGG AGGATGAGAG     100
GAGCACCCAG TCGTACCAGG GAGGTGAGGC CGCCGAGCAG GTGGAGGTGA     150
CGGACAGGGG CCTCCTCGGC AACCTCCTCG GCAAGAAGAA GGCTGAGGAG     200
GACAAGGAGA AGGAGGAGGA GCTGGTCACC GGCATGGAGA AGGTCTCCGT     250
GGAAGAGCCC GAGGTCAAGA AGGAGGAGCA CGAGGATGGC GAGAAGAAGG     300
AGACCCTCTT CTCCAAGCTG CACCGATCCA GCTCCAGCTC CAGCTCGTCT     350
AGTGACGAGG AAGAAGAGGA GGTGATCGAT GACAACGGCG AGGTGATCAA     400
GAGGAAGAAG AAGAAGGGGC TCAAGGAAAA GCTCCAGGGG AAGCTGCCCG     450
GCCACAAGGA CACCGAGGGT GAGCACGTGA CGGGGCTACC GGCACCGGCG     500
GCCCCCGCGT CTGTGCAGAC CCACGGCGGC CACCATGACA CCGACGTCGT     550
CGTCGAGAAG ATCGACGGCG ACGTGAAGAC AGAGGCGGCA CCGGCAGTGC     600
CCGAGGAGGA GAAGAAAGGC TTCTTGGAAA AGATCAAGGA GAAGCTGCCC     650
GGCGGCCACA AGAAGCCGGA GGACGCTGCT GCGGTGCCCG TCACGCACGC     700
TGCTCCAGCA CCAGTGCACG CGCCGGTGCC GGCCCCCGAG GAGGTGAGCA     750
GCCCTGACGC GAAGGAGAAG AAGGGCCTGC TGGGCAAGAT CATGGACAAG     800
CTGCCTGGTT ACCACAAGAC AGGGGAGGAG GACAAGGCCG CCGCCGCTAC     850
AGGCGAGCAC AAGCCCAGCG CTTGATCGCC GCCGTGCCCG AGACCCGTGA     900
CCGGACCTCG ATTGAATTGT TGGCGTGTGT TGTGTTTGCT TTACGTCTAA     950
GTTGGTGTCA AGGTGGGAGG GGTTGATCGT CTTTGAAGGT CCGGTCCGTG    1000
AAGCCCGTTC AGTGACGGGT GCTTCTGTTT CAGTTTGGTT CAGAGTCAGG    1050
TCCTGGATGT TGTCAAGTTT GTTTACTTAT GGGCACTTGT GTATTGGTTT    1100
ATTGCTGGGC ATTATGCCTT GATATTAAAG ATTTCC                   1136
```

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 262
(B) TYPE: Amino acid
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Protein

(vi) ORIGINAL SOURCE: Triticum Aestivum L.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6

```
Met Glu Asp Glu Arg Ser Thr Gln Ser Tyr
                5                  10
Gln Gly Gly Glu Ala Ala Glu Gln Val Glu
               15                  20
Val Thr Asp Arg Gly Leu Leu Gly Asn Leu
               25                  30
Leu Gly Lys Lys Lys Ala Glu Glu Asp Lys
```

```
                    35                  40
Glu Lys Glu Glu Glu Leu Val Thr Gly Met
                    45                  50
Glu Lys Val Ser Val Glu Glu Pro Glu Val
                    55                  60
Lys Lys Glu Glu His Glu Asp Gly Glu Lys
                    65                  70
Lys Glu Thr Leu Phe Ser Lys Leu His Arg
                    75                  80
Ser Ser Ser Ser Ser Ser Ser Ser Ser Asp
                    85                  90
Glu Glu Glu Glu Glu Val Ile Asp Asp Asn
                    95                 100
Gly Glu Val Ile Lys Arg Lys Lys Lys Lys
                   105                 110
Gly Leu Lys Glu Lys Leu Gln Gly Lys Leu
                   115                 120
Pro Gly His Lys Asp Thr Glu Gly Glu His
                   125                 130
Val Thr Gly Leu Pro Ala Pro Ala Ala Pro
                   135                 140
Ala Ser Val Gln Thr His Gly Gly His His
                   145                 150
Asp Thr Asp Val Val Val Glu Lys Ile Asp
                   155                 160
Gly Asp Val Lys Thr Glu Ala Ala Pro Ala
                   165                 170
Val Pro Glu Glu Glu Lys Lys Gly Phe Leu
                   175                 180
Glu Lys Ile Lys Glu Lys Leu Pro Gly Gly
                   185                 190
His Lys Lys Pro Glu Asp Ala Ala Ala Val
                   195                 200
Pro Val Thr His Ala Ala Pro Ala Pro Val
                   205                 210
His Ala Pro Val Pro Ala Pro Glu Glu Val
                   215                 220
Ser Ser Pro Asp Ala Lys Glu Lys Lys Gly
                   225                 230
Leu Leu Gly Lys Ile Met Asp Lys Leu Pro
                   235                 240
Gly Tyr His Lys Thr Gly Glu Glu Asp Lys
                   245                 250
Ala Ala Ala Ala Thr Gly Glu His Lys Pro
                   255                 260
Ser Ala
```

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A protein, isolated from cold tolerant wheat plants, which expression in said plants is inducible by low temperature, having the following amino acid sequence or conservative amino acid substitutions thereof:

```
Met Ile Ser Ala Ile Gln Ile Arg Met Val
                  5                  10
Gln Arg Cys Ala Arg Leu Pro Phe Pro Ile
                 15                  20
Gly Thr His Pro Val Ser Phe His Asn Leu
                 25                  30
Pro Tyr Pro Pro Thr His Gln Gln Phe Phe
                 35                  40
Tyr Arg Pro Met Ala Ser Ser Ser Val Leu
                 45                  50
Leu Gly Ala Ser Ala Thr Ala Ala Leu Thr
                 55                  60
Gly Thr Pro Ala Gly Lys Ala Leu Pro Arg
                 65                  70
Pro Cys Phe Leu Ala Ala Arg Pro Arg Thr
                 75                  80
Val Ser Gly Gly Arg Leu Cys Leu Gln Asn
                 85                  90
Ala Pro Arg Ala Thr Pro Ala Tyr Asn Asp
                 95                 100
Ala Ala Asp Ala Thr Asp Lys Ala Ile Asp
                105                 110
Gly Val Lys Gly Val Ala Asp Glu Leu Lys
                115                 120
Lys Gly Val Ala Glu Ala Ala Glu Ala Val
                125                 130
Ser Gly Asn Thr Glu Lys Ala Ala Glu Glu
                135                 140
Ala Gly Lys Gly Ala Ser Glu Val Asp Ala
                145                 150
Lys Ala Lys Asp Phe Gly Glu Gln Ala Lys
                155                 160
Lys Ala Thr Glu Glu Ala Trp Asp Gly Ala
                165                 170
```

```
Lys Asp Ala Ala Gln Gly Ile Thr Asp Lys
                175                 180
Val Ala Ala Ala Ala Lys Lys Glu Ala Ser
                185                 190
```

2. A protein, isolated from cold tolerant wheat plants, which expression in said plants is inducible by low temperature, having the following amino acid sequence or conservative amino acid substitutions thereof:

```
Met Glu Asp Glu Arg Ser Thr Gln Ser Tyr
                  5                  10
Gln Gly Gly Glu Ala Ala Glu Gln Val Glu
                 15                  20
Val Thr Asp Arg Gly Leu Leu Gly Asn Leu
                 25                  30
Leu Gly Lys Lys Lys Ala Glu Glu Asp Lys
                 35                  40
Glu Lys Glu Glu Glu Leu Val Thr Gly Met
                 45                  50
Glu Lys Val Ser Val Glu Glu Pro Glu Val
                 55                  60
Lys Lys Glu Glu His Glu Asp Gly Glu Lys
                 65                  70
Lys Glu Thr Leu Phe Ser Lys Leu His Arg
                 75                  80
Ser Ser Ser Ser Ser Ser Ser Ser Ser Asp
                 85                  90
Glu Glu Glu Glu Glu Val Ile Asp Asp Asn
                 95                 100
Gly Glu Val Ile Lys Arg Lys Lys Lys Lys
                105                 110
Gly Leu Lys Glu Lys Leu Gln Gly Lys Leu
                115                 120
Pro Gly His Lys Asp Thr Glu Gly Glu His
                125                 130
Val Thr Gly Leu Pro Ala Pro Ala Ala Pro
                135                 140
Ala Ser Val Gln Thr His Gly Gly His His
                145                 150
Asp Thr Asp Val Val Val Glu Lys Ile Asp
                155                 160
Gly Asp Val Lys Thr Glu Ala Ala Pro Ala
                165                 170
Val Pro Glu Glu Glu Lys Lys Gly Phe Leu
```

```
                    175                 180
Glu Lys Ile Lys Glu Lys Leu Pro Gly Gly
                    185                 190
His Lys Lys Pro Glu Asp Ala Ala Ala Val
                    195                 200
Pro Val Thr His Ala Ala Pro Ala Pro Val
                    205                 210
His Ala Pro Val Pro Ala Pro Glu Glu Val
                    215                 220
Ser Ser Pro Asp Ala Lys Glu Lys Lys Gly
                    225                 230
Leu Leu Gly Lys Ile Met Asp Lys Leu Pro
                    235                 240
Gly Tyr His Lys Thr Gly Glu Glu Asp Lys
                    245                 250
Ala Ala Ala Ala Thr Gly Glu His Lys Pro
                    255                 260
Ser Ala
```

3. A protein, isolated from cold tolerant wheat plants, which expression in said plants is inducible by low temperature, having the following amino acid sequence or conservative amino acid substitutions thereof:

```
Met Glu Asn Gln Ala His Ile Ala Gly Glu
                      5                  10
Lys Lys Gly Ile Met Glu Lys Ile Lys Glu
                     15                  20
Lys Leu Pro Gly Gly His Gly Asp His Lys
                     25                  30
Glu Thr Ala Gly Thr His Gly His Pro Gly
                     35                  40
Thr Ala Thr His Gly Ala Pro Ala Thr Gly
                     45                  50
Gly Ala Tyr Gly Gln Gln Gly His Ala Gly
                     55                  60
Thr Thr Gly Thr Gly Leu His Gly Ala His
                     65                  70
Ala Gly Glu Lys Lys Gly Val Met Glu Asn
                     75                  80
Ile Lys Asp Lys Leu Pro Gly Gly His Gln
                     85                  90
Asp His Gln Gln Thr Gly Gly Thr Tyr Gly
                     95                 100
Gln Gln Gly Thr His Gly Thr Ala Thr His
                    105                 110
Gly Thr Pro Ala Thr Gly Gly Thr Tyr Gly
```

```
                    115                 120
Gln Gln Gly His Thr Gly Thr Ala Thr His
                    125                 130
Gly Thr Pro Ala Thr Gly Gly Thr Tyr Gly
                    135                 140
Glu Gln Gly His Thr Gly Val Thr Gly Thr
                    145                 150
Gly Thr His Gly Thr Gly Glu Lys Lys Gly
                    155                 160
Val Met Glu Asn Ile Lys Glu Lys Leu Pro
                    165                 170
Gly Gly His Gly Asp His Gln Gln Thr Gly
                    175                 180
Gly Thr Tyr Gly Gln Gln Gly His Thr Gly
                    185                 190
Thr Ala Thr His Gly Thr Pro Ala Gly Gly
                    195                 200
Gly Thr Tyr Glu Gln His Gly His Thr Gly
                    205                 210
Met Thr Gly Thr Gly Thr His Gly Thr Gly
                    215                 220
Glu Lys Lys Gly Val Met Glu Asn Ile Lys
                    225                 230
Asp Lys Leu Pro Gly Gly His Gly Asp His
                    235                 240
Gln Gln Thr Gly Gly Thr Tyr Gly Gln Gln
                    245                 250
Gly His Thr Gly Thr Ala Thr Gln Gly Thr
                    255                 260
Pro Ala Gly Gly Gly Thr Tyr Glu Gln His
                    265                 270
Gly His Thr Gly Met Thr Gly Ala Gly Thr
                    275                 280
His Ser Thr Gly Glu Lys Lys Gly Val Met
                    285                 290
Glu Asn Ile Lys Glu Lys Leu Pro Gly Gly
                    295                 300
His Ser Asp His Gln Gln Thr Gly Gly Ala
                    305                 310
Tyr Gly Gln Gln Gly His Thr Gly Thr Arg
                    315                 320
His Met Ala Pro Leu Pro Ala Gly Thr Tyr
                    325                 330
Gly Gln His Gly His Ala Gly Val Ile Gly
                    335                 340
Thr Glu Thr His Gly Thr Thr Ala Thr Gly
                    345                 350
Gly Thr His Gly Gln His Gly His Thr Gly
                    355                 360
Thr Thr Gly Thr Gly Thr His Gly Ser Asp
                    365                 370
Gly Ile Gly Glu Lys Lys Ser Leu Met Asp
```

375 380
Lys Ile Lys Asp Lys Leu Pro Gly Gln His
385 390

4. A nucleic acid encoding the protein of Claim 1.

5. A nucleic acid encoding the protein of Claim 2.

6. A nucleic acid encoding the protein of Claim 3.

7. A nucleic acid according to Claim 4 having the following nucleic acid sequence, said protein being encoded by nucleotides numbered 205 to 774 of said nucleic acid sequence:

TTTTTTTTTG CGACCAAAAT GAACAAGTAA ATTTACTCCC TCACAAGCAT 50
ATGCAAATAT ATTCCACCAA GAATATTAGT CGGTCCTCGC TATCAACCAC 100
ATCTAAAACC ATGTCAACGA ATGGAAACAA CACCACCTTA AAAGTATCCA 150
CACGAGAAGG CTCCTTATAT TTGTATTAAC AGAAGAGCAA AAAGATATAG 200
CTGTATGATT TCAGCGATCC AAATCCGCAT GGTGCAGCGA TGCGCAAGAC 250
TACCATTTCC AATCGGCACA CATCCTGTCT CCTTCCACAA CCTACCCTAC 300
CCACCCATCC ATCAGCAGTT TTTCTATCGA CCAATGGCTT CTTCTTCCGT 350
GCTGCTCGGA GCCTCGGCCA CGGCCGCGCT CACCGGCACC CCGGCAGGCA 400
AGGCCCTTCC CCGGCCTTGC TTCCTCGCCG CTCGCCCGCG CACCGTGAGC 450
GGTGGCCGTC TCTGCCTGCA GAACGCTCCA AGGGCGACTC CGGCGTACAA 500
CGACGCTGCG GATGCCACCG ACAAGGCCAT CGACGGCGTG AAGGGGGTGG 550
CCGACGAGTT GAAGAAGGGC GTGGCGGAGG CTGCGGAGGC CGTCTCGGGC 600

AACACCGAGA AGGCCGCGGA GGAAGCCGGC AAGGGCGCGA GCGAGGTGGA 650

CGCGAAGGCC AAGGACTTCG GCGAGCAGGC GAAGAAGGCG ACGGAGGAGG 700

CGTGGGACGG CGCCAAGGAC GCCGCACAGG GCATCACGGA CAAAGTCGCC 750

GCCGCGGCCA AAAAGGAAGC TAGCTAAGCT AACACTACGT TGACTAGTCC 800

GATCTGTATC GCTCAATTCA TTTTCCATTG TAAGGAATGC ATATACGTAT 850

TTCGGTACAA GAGATAAGAT AGCTGTATTT ATTTTCTGTG ATATAGGATT 900

ACCGCACTGT TAATGTCAAA CGCAATAAAG AAAATGATTT TTY 943

8. A nucleic acid according to Claim 5 having the following nucleic acid sequence, said protein being encoded by nucleotides numbered 87 to 872 of said nucleic acid sequence:

AAAAGCCACA AGCCAAGAAC CAATACTTGA TCTGTTGTTT CCTTTAGCTC 50

CCGGAAGACT TTTAGCTGCA CCGATCGATC TCGATCATGG AGGATGAGAG 100

GAGCACCCAG TCGTACCAGG GAGGTGAGGC CGCCGAGCAG GTGGAGGTGA 150

CGGACAGGGG CCTCCTCGGC AACCTCCTCG GCAAGAAGAA GGCTGAGGAG 200

GACAAGGAGA AGGAGGAGGA GCTGGTCACC GGCATGGAGA AGGTCTCCGT 250

GGAAGAGCCC GAGGTCAAGA AGGAGGAGCA CGAGGATGGC GAGAAGAAGG 300

AGACCCTCTT CTCCAAGCTG CACCGATCCA GCTCCAGCTC CAGCTCGTCT 350

AGTGACGAGG AAGAAGAGGA GGTGATCGAT GACAACGGCG AGGTGATCAA 400

GAGGAAGAAG AAGAAGGGGC TCAAGGAAAA GCTCCAGGGG AAGCTGCCCG 450

GCCACAAGGA CACCGAGGGT GAGCACGTGA CGGGGCTACC GGCACCGGCG 500

GCCCCCGCGT CTGTGCAGAC CCACGGCGGC CACCATGACA CCGACGTCGT 550

CGTCGAGAAG ATCGACGGCG ACGTGAAGAC AGAGGCGGCA CCGGCAGTGC 600

CCGAGGAGGA GAAGAAAGGC TTCTTGGAAA AGATCAAGGA GAAGCTGCCC 650

GGCGGCCACA AGAAGCCGGA GGACGCTGCT GCGGTGCCCG TCACGCACGC 700

TGCTCCAGCA CCAGTGCACG CGCCGGTGCC GGCCCCCGAG GAGGTGAGCA 750

GCCCTGACGC GAAGGAGAAG AAGGGCCTGC TGGGCAAGAT CATGGACAAG 800

CTGCCTGGTT ACCACAAGAC AGGGGAGGAG GACAAGGCCG CCGCCGCTAC 850

AGGCGAGCAC AAGCCCAGCG CTTGATCGCC GCCGTGCCCG AGACCCGTGA 900

CCGGACCTCG ATTGAATTGT TGGCGTGTGT TGTGTTTGCT TTACGTCTAA 950

GTTGGTGTCA AGGTGGGAGG GGTTGATCGT CTTTGAAGGT CCGGTCCGTG 1000

AAGCCCGTTC AGTGACGGGT GCTTCTGTTT CAGTTTGGTT CAGAGTCAGG 1050

TCCTGGATGT TGTCAAGTTT GTTTACTTAT GGGCACTTGT GTATTGGTTT 1100

ATTGCTGGGC ATTATGCCTT GATATTAAAG ATTTCC 1136

9. A nucleic acid according to Claim 6 having the following nucleic acid sequence, said protein being encoded by nucleotides numbered 23 to 1192 of said nucleic acid sequence:

CGAGTGAGGA GCTCAGCGCA AGATGGAGAA CCAGGCACAC ATCGCCGGCG 50

AGAAGAAGGG CATCATGGAG AAGATCAAGG AGAAGCTCCC CGGCGGCCAC 100

GGCGACCACA AGGAGACCGC TGGTACCCAC GGGCACCCCG GCACGGCGAC 150

GCATGGTGCC CCGGCCACTG GTGGTGCCTA CGGGCAGCAG GGTCACGCTG 200

GAACCACCGG CACGGGGTTG CATGGCGCCC ACGCCGGCGA GAAGAAGGGC 250

GTCATGGAGA ACATCAAGGA CAAGCTCCCT GGTGGCCACC AGGACCACCA 300

GCAGACTGGT GGTACCTATG GGCAGCAGGG ACACACCGGC ACGGCGACGC 350

ATGGCACCCC GGCGACCGGT GGCACCTATG GGCAGCAGGG ACATACCGGC 400

ACAGCGACGC ATGGCACCCC GGCGACCGGT GGCACCTATG GGGAGCAGGG 450

ACACACCGGA GTGACTGGCA CGGGGACGCA CGGCACCGGC GAGAAGAAGG 500

CGGTGACCAC GAACATCAAG GAGAAGCTCC CTGGTGGCCA CGGCACCGGC 550

CAGCAGACCG GTGGTACCTA CGGGCAGCAG GGACACACCG GCACGGCGAC 600

GCATGGCACC CCGGCCGGGG GCGGCACCTA TGAGCAGCAC GGACACACCG 650

GGATGACCGG CACAGGGACA CACGGCACTG GCGAGAAGAA AGGCGTCATG 700

GAGAACATCA AGGACAAGCT CCCTGGTGGC CACGGAGATC ACCAGCAGAC 750

CGGTGGCACC TACGGGCAGC AGGGACACAC CGGCACGGCG ACACAGGGCA 800

CCCCGGCCGG CGGCGGCACC TATGAGCAGC ATGGACACAC CGGGATGACC 850

GGCGCGGGGA CACACAGCAC TGGCGAGAAG AAGGGCGTCA TGGAGAACAT 900

CAAGGAAAAG CTCCCTGGTG GCCACAGTGA CCACCAGCAG ACCGGTGGAG 950

CCTACGGGCA GCAGGGACAC ACCGGCACGC GACACATGGC ACCCCTGCCG 1000

GCGGGCACCT ACGGGCAGCA TGGACACGCT GGAGTGATCG GCACGGAGAC 1050

GCATGGCACC ACGGCCACCG GCGGCACCCA TGGGCAGCAC GGACACACCG 1100

GAACGACTGG CACTGGGACA CACGGCTCCG ACGGGATCGG CGAGAAGAAG 1150

AGCCTCATGG ACAAGATCAA GGATAAGCTG CCTGGACAGC ACTGAGCCCG 1200

GTCTGCCCGC GGCCGCTACC CTTGCAGAAT AATAACCCCA CCGTGTATAA 1250

GTTAATTGAG TCTAGTTCAC CTAGCTCACT TGGTCGTTGG AGGAGAGAAT 1300

GTATTATGTA TCTTGGTTTA AGTTTTCACG GACAACAGTG TGTTCACAGT 1350

TTTCTTCTGT TTACACTCTG TAGTGCAAAT TCGTTTAAGT TTTCACGGAC 1400

AACAGTGTGT TCACAGTTTT CTTCTGTTTA CACTCTGTAG TGCAAATTTC 1450

GTTTTTGTTC TTTTTTTTTT TGTCCATCTT ATCCAAGAGA CAGACGCAGC 1500

GAAAAAAAAA AAAAAAAAAA AA 1522

10. The use of the protein of Claim 2 as a protective agent against freezing.

11. The use of the protein of Claim 3 as a protective agent against freezing.

12. A method of producing the protein Wcs120 and substituted derivatives thereof, which comprises the following steps:

cloning the nucleic acid of Claim 6 in a plasmidic vector which is suitable to conduct the expression of said protein in a host cell compatible with said vector;

allowing the expression of the cloned nucleic acid in said host cell; and

purifying said expressed protein.

13. A method of producing the protein Wcor410 and substituted derivatives thereof, which comprises the following steps:

cloning the nucleic acid of Claim 5 in a plasmidic vector which is suitable to conduct the expression of said protein in a host cell compatible with said vector;

allowing the expression of the cloned nucleic acid in said host cell; and

purifying said expressed protein.

14. The method of Claim 12 wherein said vector is selected from pUC9 and pET vectors.

15. The method of Claim 13 wherein said vector is selected from pUC9 and pET vectors.

16. The method of Claim 14 wherein said host cell is E. coli.

17. The method of Claim 15 wherein said host cell is E. coli.

18. A DNA molecule which is homologous to the nucleic acid of Claim 7.

19. A DNA molecule which is homologous to the nucleic acid of Claim 8.

20. A DNA molecule which is homologous to the nucleic acid of Claim 9.

21. A protein which is immunologically related to the Wcs120 protein, as determined by its cross-reactivity with an antibody raised against the Wcs120 protein.

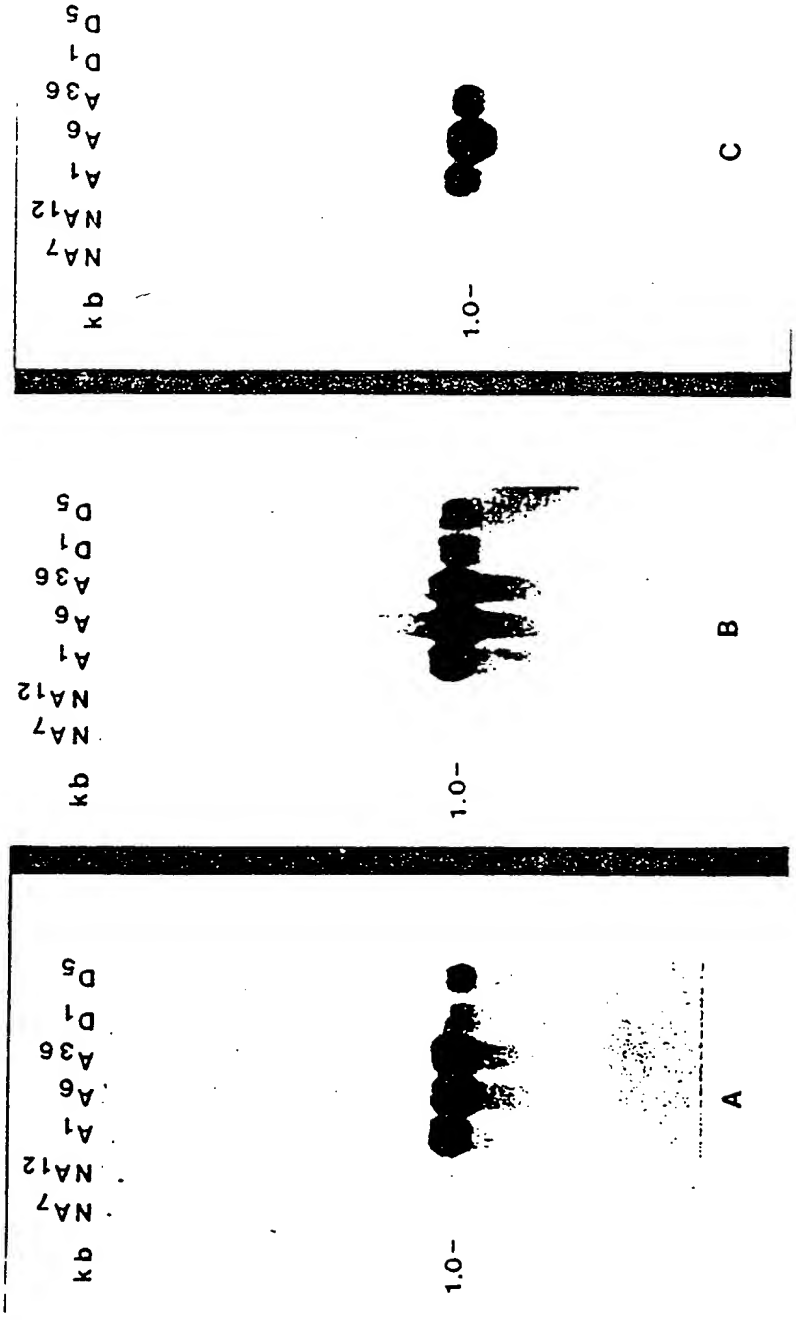

Fig. 1

Goudreau Gage Dubuc & Martineau Walker

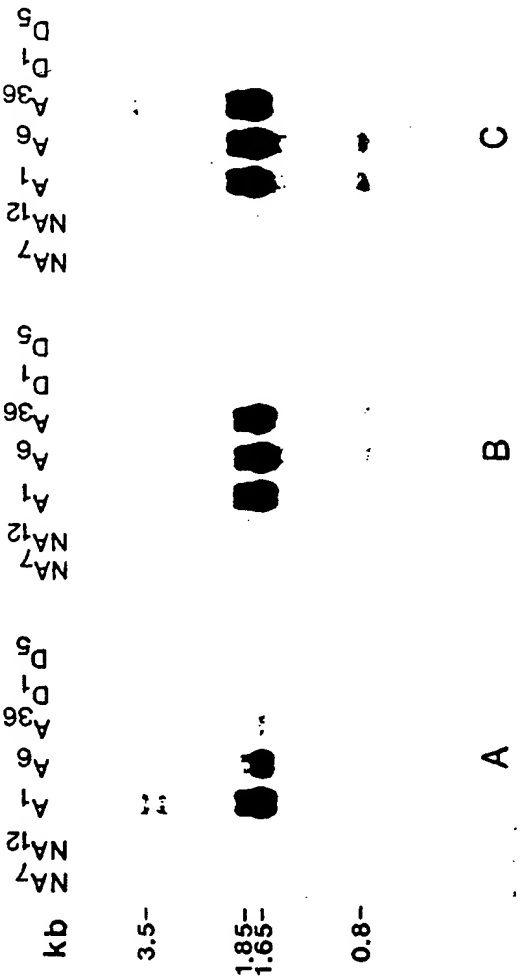

Fig. 2

Goudreau Gage Dubuc & Martineau Walker

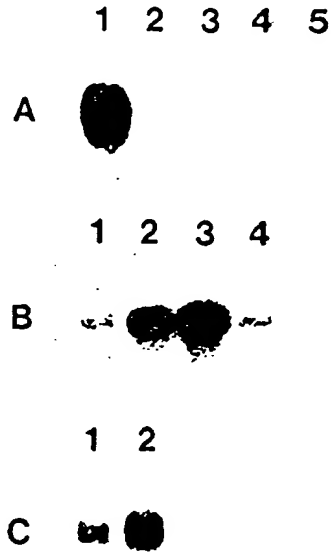

Fig. 3

Goudreau Gage Dubuc & Hinchman Walker,

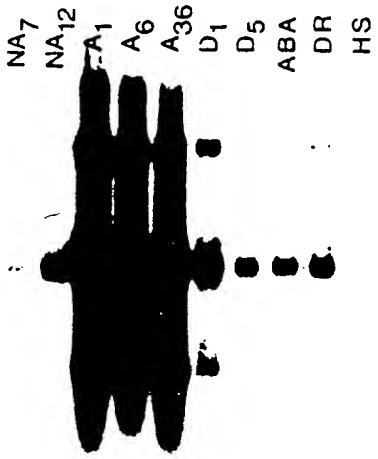

Fig. 4

Gudrun Page Dubus & Patricia Walker

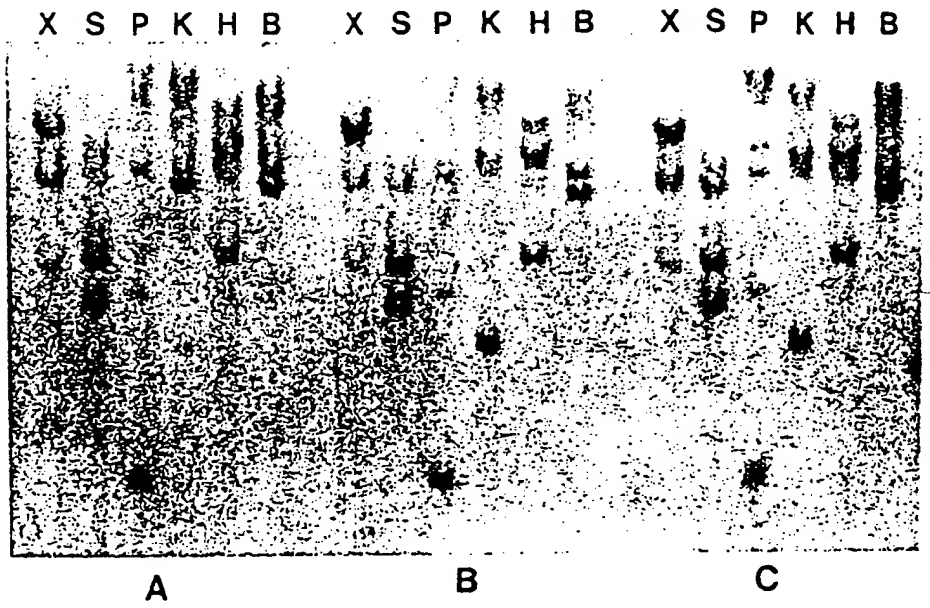

Fig. 5

Goudreau Gage Dubuc & Martineau Walker

Fig. 6

Goudreau Gage Dubuc & Martineau Walker

```
   1  ttttttttgcgaccaaaatgaacaagtaaatttactccctcacaagc
  49  atatgcaaatatattccaccaagaatattagtcggtcctcgctatcaa
  97  ccacatctaaaaccatgtcaacgaatggaaacaacaccaccttaaaag
 145  tatccacacgagaaggctccttatatttgtattaacagaagagcaaaa
 193  agatatagctgtatgatttcagcgatccaaatccgcatggtgcagcga
 (1)                M  I  S  A  I  Q  I  R  M  V  Q  R
                    α  α  α  α  α  α  α  α  α  α  α  α
 241  tgcgcaagactaccatttccaatcggcacacatcctgtctccttccac
 (13) C  A  R  L  P  F  P  I  G  T  H  P  V  S  F  H
      α  ⌟  ⌟  ◘  ⌟  ◘  β  β  ◘  ◘  ◘  β  β  β  β  β
 289  aacctaccctacccacccatccatcagcagttttctatcgaccaatg
 (29) N  L  P  Y  P  P  T  H  Q  Q  F  F  Y  R  P  M
      ◘  ◘  ⌟  ◘  ◘  ◘  ◘  ◘  ◘  β  β  β  β  ◘  α  ⌟
 337  gcttcttcttccgtgctgctcggagcctcggccacggccgcgctcacc
 (45) A  S  S  S  V  L  L  G  A  S  A  T  A  A  L  T
      ⌟  ⌟  ⌟  β  β  β  β  β  α  α  α  β  β  β  β  β
 385  ggcaccccggcaggcaaggcccttccccggccttgcttcctcgccgct
 (61) G  T  P  A  G  K  A  L  P  R  P  C  F  L  A  A
      ◘  ◘  ◘  ◘  ◘  ◘  ◘  ◘  ◘  ◘  ⌟  α  α  α  α  α
 433  cgcccgcgcaccgtgagcggtggccgtctctgcctgcagaacgctcca
 (77) R  P  R  T  V  S  G  G  R  L  C  L  Q  N  A  P
      ◘  ⌟  β  β  β  β  ⌟  β  β  β  β  β  ⌟  ⌟  ◘  ◘
 481  agggcgactccggcgtacaacgacgctgcggatgccaccgacaaggcc
 (93) R  A  T  P  A  Y  N  D  A  A  D  A  T  D  K  A
      ⌟  ◘  ◘  ◘  ◘  ◘  ◘  α  α  α  α  α  α  α  α  α
 529  atcgacggcgtgaaggggtggccgacgagttgaagaagggcgtggcg
 (109) I  D  G  V  K  G  V  A  D  E  L  K  K  G  V  A
      α  α  α  α  α  α  α  α  α  α  α  α  α  α  α  α
 577  gaggctgcggaggccgtctcgggcaacaccgagaaggccgcggaggaa
 (125) E  A  A  E  A  V  S  G  N  T  E  K  A  A  E  E
      α  α  α  α  α  α  α  α  α  α  α  α  α  α  α  α
 625  gccggcaagggcgcgagcgaggtggacgcgaaggccaaggacttcggc
 (141) A  G  K  G  A  S  E  V  D  A  K  A  K  D  F  G
      α  α  α  α  α  α  α  α  α  α  α  α  α  α  α  α
 673  gagcaggcgaagaaggcgacggaggaggcgtgggacggcgccaaggac
 (157) E  Q  A  K  K  A  T  E  E  A  W  D  G  A  K  D
      α  α  α  α  α  α  α  α  α  α  α  α  α  α  α  α
 721  gccgcacagggcatcacggacaaagtcgccgccgcggccaaaaaggaa
 (173) A  A  Q  G  I  T  D  K  V  A  A  A  A  K  K  E
      α  α  α  α  α  α  α  α  α  α  α  α  α  α  α  α
 769  gctagctaagctaacactacgttgactagtccgatctgtatcgctcaa
 (189) A  S
      α  α
 817  ttcattttccattgtaaggaatgcatatacgtatttcggtacaagaga
 865  taagatagctgtatttatttttctgtgatataggattaccgcactgtta
 913  atgtcaaacgcaataaagaaaatgattttt
```

Fig. 7

Goudreau Gage Dubuc & Hartman Walker

```
AAAAGCCACA AGCCAAGAAC CAATACTTGA TCTGTTGTTT CCTTTAGCTC        50
CCGGAAGACT TTTAGCTGCA CCGATCGATC TCGATC ATG GAG GAT GAG        98
                                        Met Glu Asp Glu

AGG AGC ACC CAG TCG TAC CAG GGA GGT GAG GCC GCC GAG CAG       140
Arg Ser Thr Gln Ser Tyr Gln Gly Gly Glu Ala Ala Glu Gln
  5                  10                  15
GTG GAG GTG ACG GAC AGG GGC CTC CTC GGC AAC CTC CTC GGC       182
Val Glu Val Thr Asp Arg Gly Leu Leu Gly Asn Leu Leu Gly
     20                  25                  30
AAG AAG AAG GCT GAG GAG GAC AAG GAG AAG GAG GAG GAG CTG       224
Lys Lys Lys Ala Glu Glu Asp Lys Glu Lys Glu Glu Glu Leu
         35                  40                  45
GTC ACC GGC ATG GAG AAG GTC TCC GTG GAA GAG CCC GAG GTC       266
Val Thr Gly Met Glu Lys Val Ser Val Glu Glu Pro Glu Val
             50                  55                  60
AAG AAG GAG GAG CAC GAG GAT GGC GAG AAG AAG GAG ACC CTC       308
Lys Lys Glu Glu His Glu Asp Gly Glu Lys Lys Glu Thr Leu
                 65                  70
TTC TCC AAG CTG CAC CGA TCC AGC TCC AGC TCC AGC TCG TCT       350
Phe Ser Lys Leu His Arg Ser Ser Ser Ser Ser Ser Ser Ser
 75                  80                  85
AGT GAC GAG GAA GAA GAG GAG GTG ATC GAT GAC AAC GGC GAG       392
Ser Asp Glu Glu Glu Glu Glu Val Ile Asp Asp Asn Gly Glu
     90                  95                 100
GTG ATC AAG AGG AAG AAG AAG AAG GGG CTC AAG GAA AAG CTC       434
Val Ile Lys Arg Lys Lys Lys Lys Gly Leu Lys Glu Lys Leu
         105                 110                 115
CAG GGG AAG CTG CCC GGC CAC AAG GAC ACC GAG GGT GAG CAC       476
Gln Gly Lys Leu Pro Gly His Lys Asp Thr Glu Gly Glu His
             120                 125                 130
GTG ACG GGG CTA CCG GCA CCG GCG GCC CCC GCG TCT GTG CAG       518
Val Thr Gly Leu Pro Ala Pro Ala Ala Pro Ala Ser Val Gln
                 135                 140
ACC CAC GGC GGC CAC CAT GAC ACC GAC GTC GTC GTC GAG AAG       560
Thr His Gly Gly His His Asp Thr Asp Val Val Val Glu Lys
145                 150                 155
ATC GAC GGC GAC GTG AAG ACA GAG GCG GCA CCG GCA GTG CCC       602
Ile Asp Gly Asp Val Lys Thr Glu Ala Ala Pro Ala Val Pro
    160                 165                 170
GAG GAG GAG AAG AAA GGC TTC TTG GAA AAG ATC AAG GAG AAG       644
Glu Glu Glu Lys Lys Gly Phe Leu Glu Lys Ile Lys Glu Lys
        175                 180                 185
CTG CCC GGC GGC CAC AAG AAG CCG GAG GAC GCT GCT GCG GTG       686
Leu Pro Gly Gly His Lys Lys Pro Glu Asp Ala Ala Ala Val
            190                 195                 200
CCC GTC ACG CAC GCT GCT CCA GCA CCA GTG CAC GCG CCG GTG       728
Pro Val Thr His Ala Ala Pro Ala Pro Val His Ala Pro Val
                205                 210
CCG GCC CCC GAG GAG GTG AGC AGC CCT GAC GCG AAG GAG AAG       770
Pro Ala Pro Glu Glu Val Ser Ser Pro Asp Ala Lys Glu Lys
215                 220                 225
```

Goudreau Gage Dubuc & Martineau Walker

```
AAG GGC CTG CTG GGC AAG ATC ATG GAC AAG CTG CCT GGT TAC      812
Lys Gly Leu Leu Gly Lys Ile Met Asp Lys Leu Pro Gly Tyr
    230                 235                 240
CAC AAG ACA GGG GAG GAG GAC AAG GCC GCC GCC GCT ACA GGC      854
His Lys Thr Gly Glu Glu Asp Lys Ala Ala Ala Ala Thr Gly
        245                 250                 255
GAG CAC AAG CCC AGC GCT TGATCGCCGC CGTGCCCGAG ACCCGTGACC      902
Glu His Lys Pro Ser Ala
            260
GGACCTCGAT TGAATTGTTG GCGTGTGTTG TGTTTGCTTT ACGTCTAAGT       952
TGGTGTCAAG GTGGGAGGGG TTGATCGTCT TTGAAGGTCC GGTCCGTGAA      1002
GCCCGTTCAG TGACGGGTGC TTCTGTTTCA GTTTGGTTCA GAGTCAGGTC      1052
CTGGATGTTG TCAAGTTTGT TTACTTATGG GCACTTGTGT ATTGGTTTAT      1102
TGCTGGGCAT TATGCCTTGA TATTAAAGAT TTCC                       1136
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Fig. 8

Goudreau Gage Dubuc & Martineau Walker

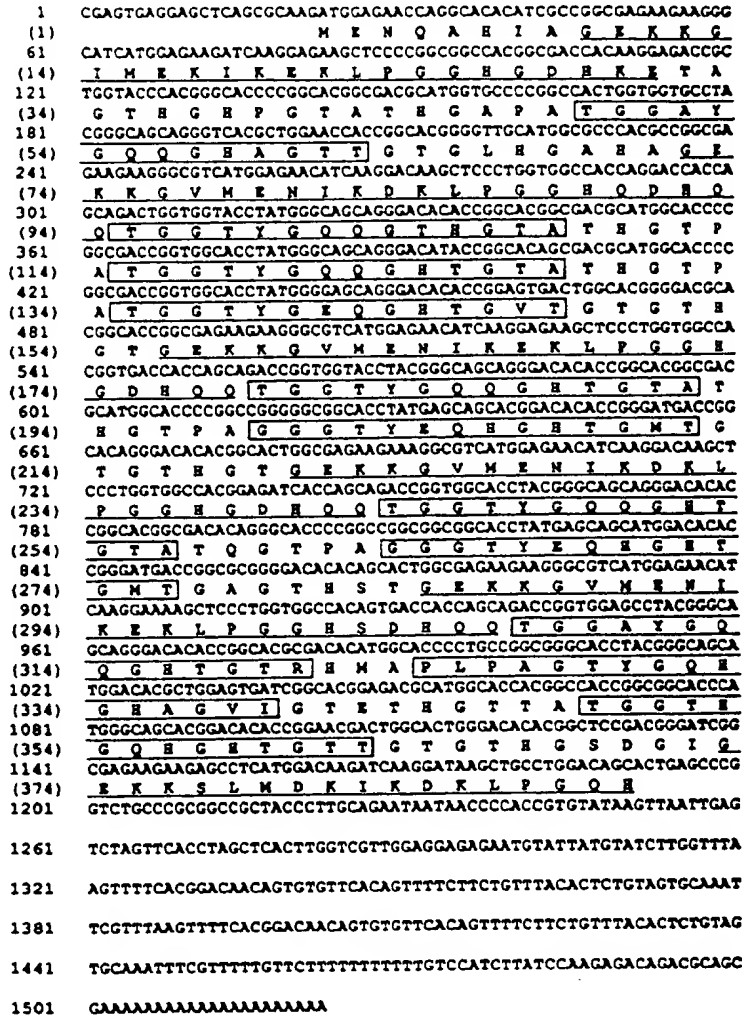


Fig. 9

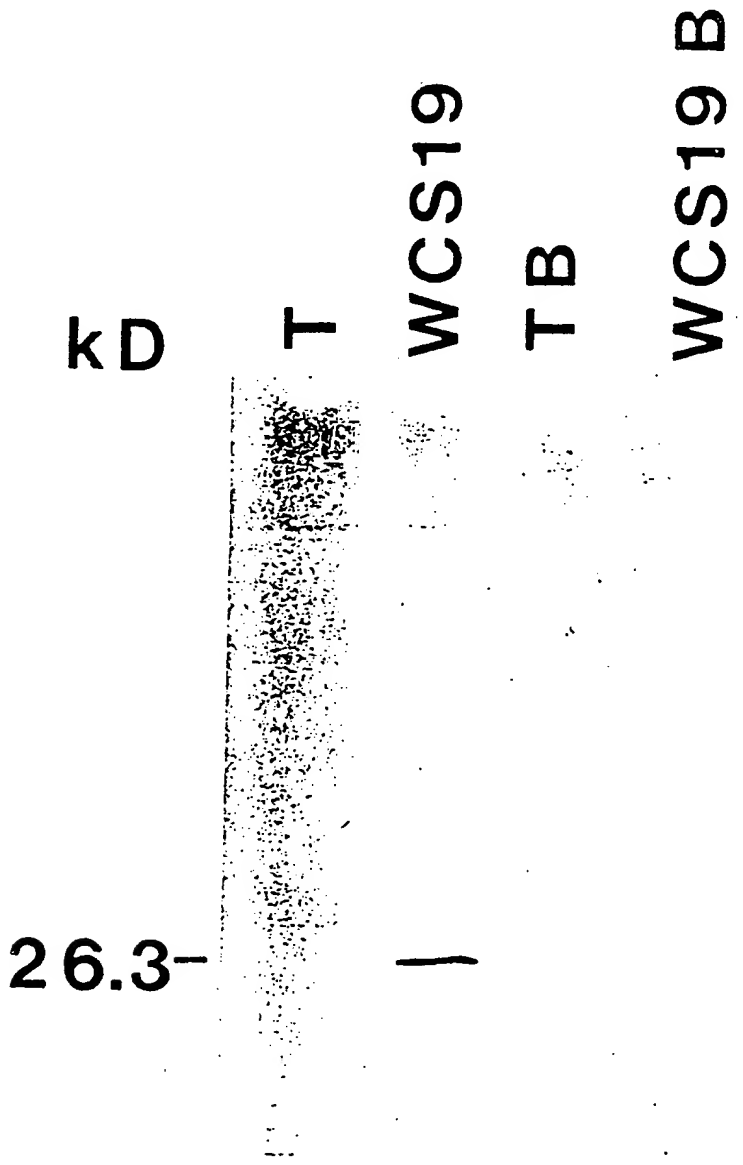


Fig. 10

Goudreau Gage Dubuc & Martineau Walker

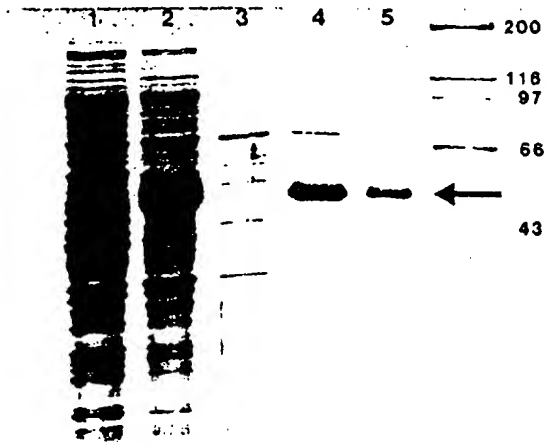


Fig. 11

Goudreau Gage Dubuc & Martineau Walker

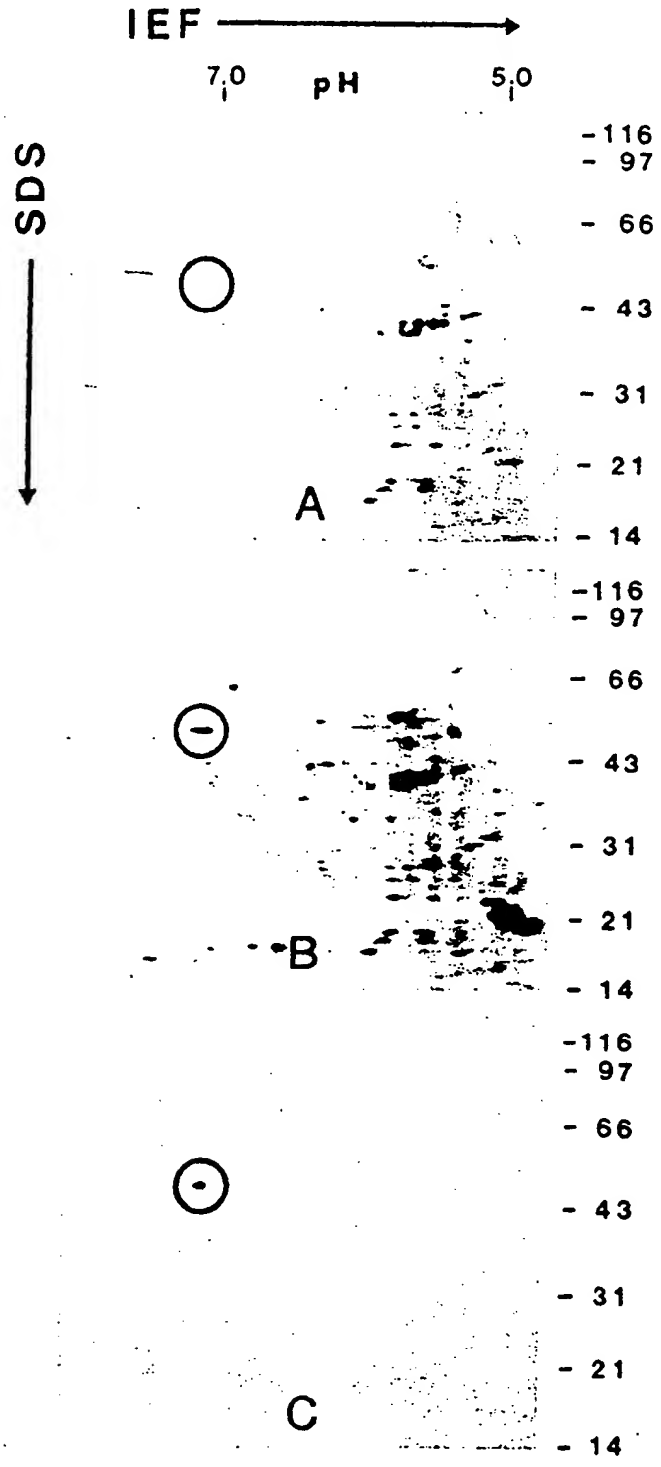


Fig. 12

Goudreau Gage Dubuc & Hamilton Walker

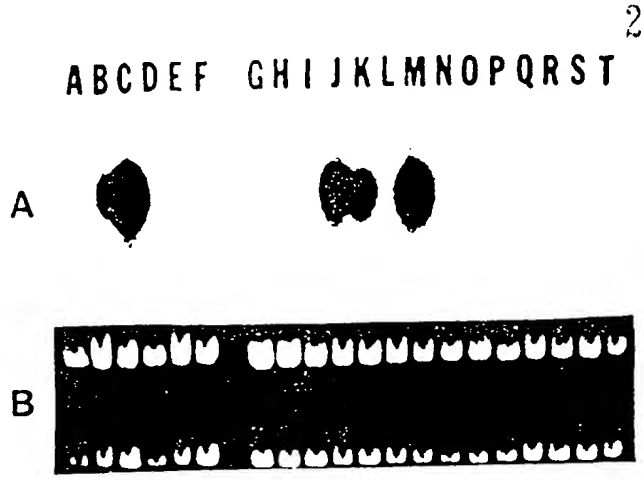


Fig. 13

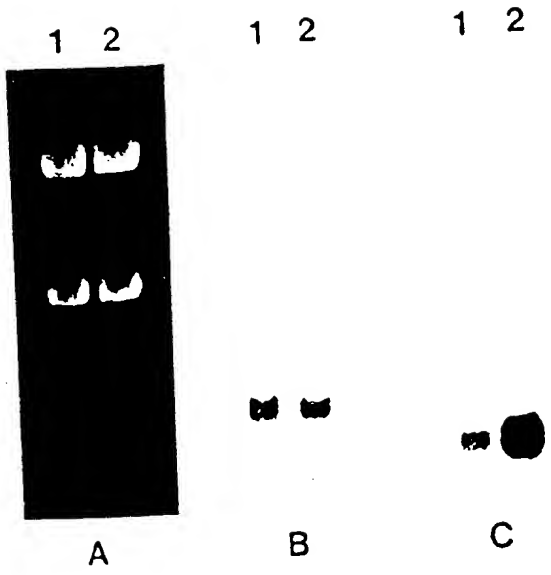
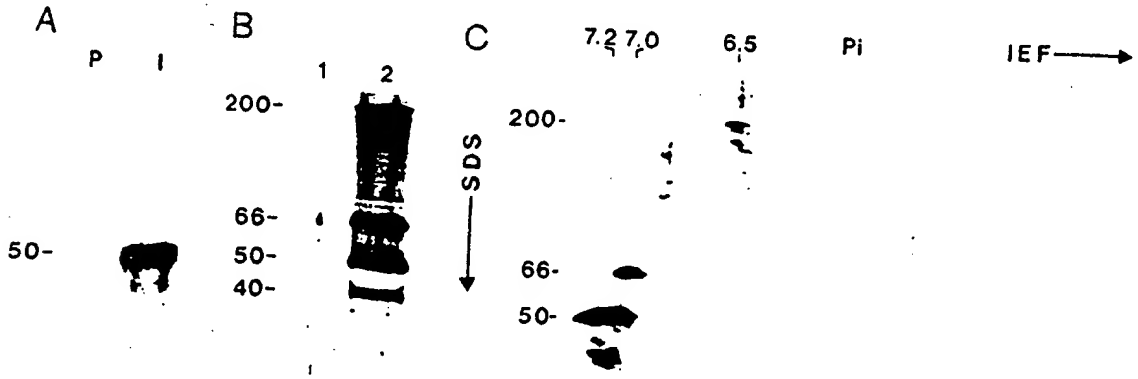


Fig. 14

Goodman Sage Dubuc & Martineau Walker



17-

Fig. 15

Goudreau Gage Dubuc & Martineau Walker

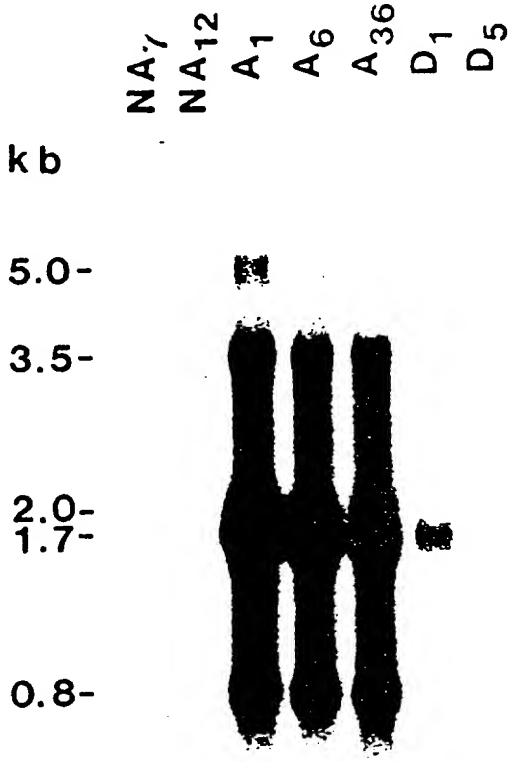


Fig. 16

Goodrean Gage Dubus & Hutchison Walker

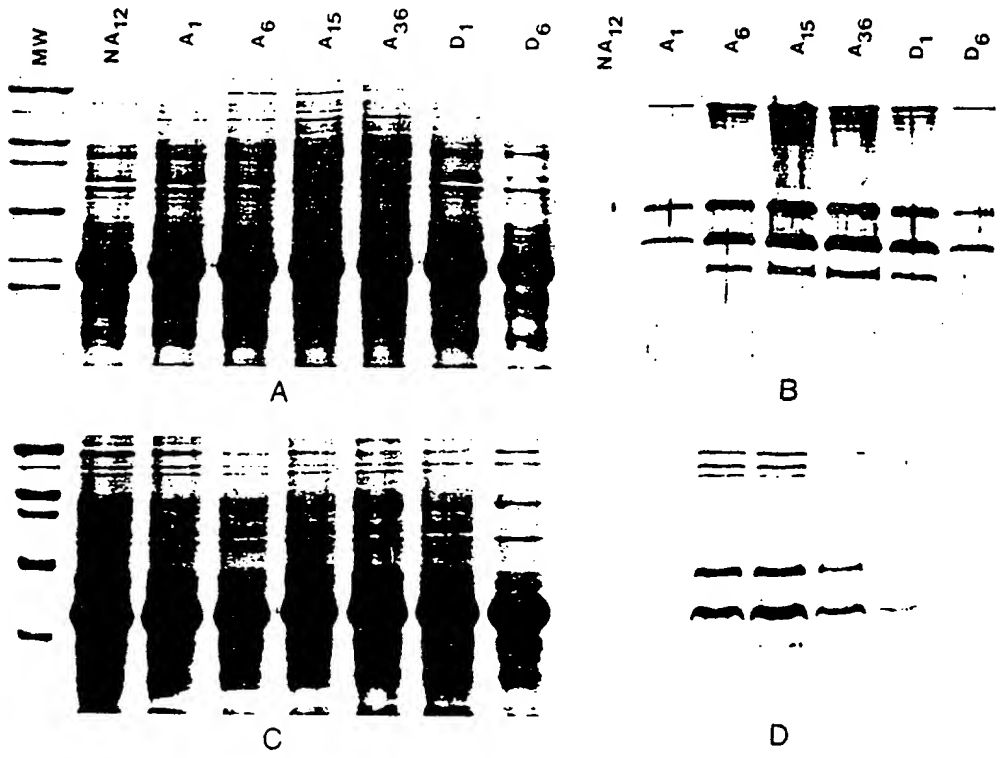


Fig. 17

Goudreau Gage Dubuc & Martineau Walker

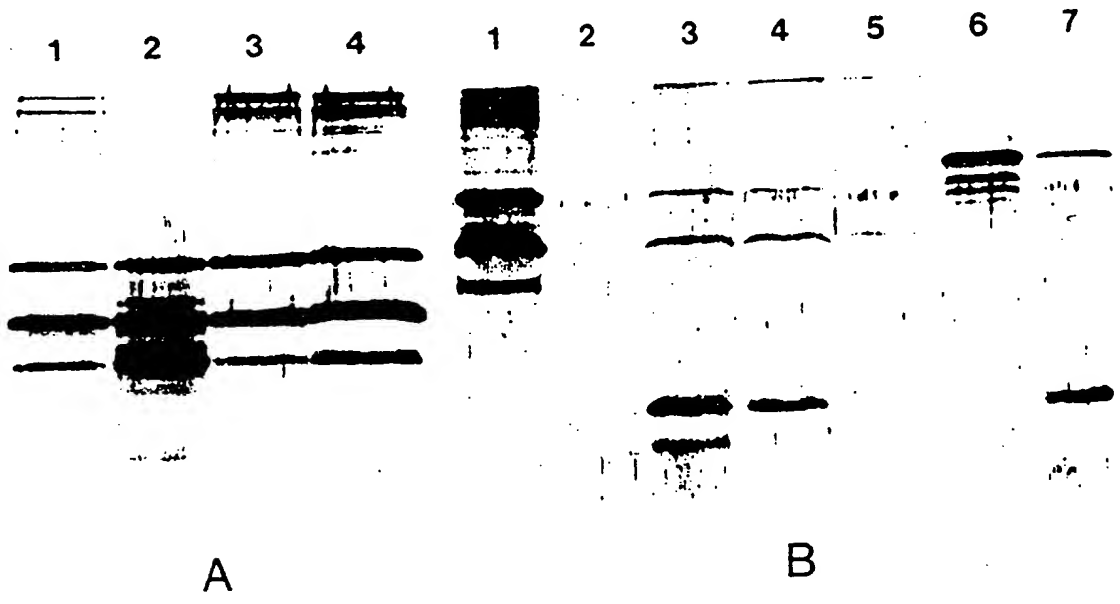


Fig. 18

Goudreau Gage Dubuc & Martineau Walker

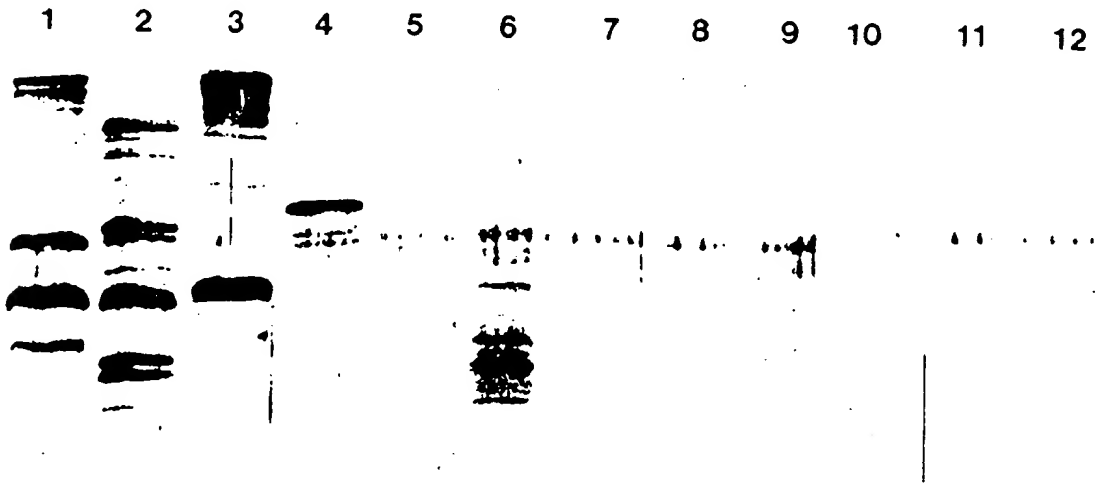


Fig. 19

Goudreau Gage Dubuc & Martineau Walker